UNIVERSITY OF ALBERTA

Regulatory Roles of Nitric Oxide in Vascular Smooth Muscle Contractility

by

Junzhi Ji

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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June 19. 1997

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Regulatory roles of nitric oxide in vascular smooth muscle contractility" submitted by Junzhi Ji in partial fulfillment of the requirements for the degree of Master of Science.

Dr. P.K.T. Pang (Supervisor)

Dr. C.G. Benishin (Co-supervisor)

Dr. R. Z. Lewanczuk

Dr. S.M.J. Dunn (External Examiner)

Dr. S. Jacobs (Committee Chair)

Date: June 17, 1997

ABSTRACT

It has been established that nitric oxide (NO) reduces vascular contractility. The mechanisms involved are not well understood. The hypothesis that NO interfered with Ca^{2+} entry and intracellular Ca^{2+} release in vascular smooth muscle (VSM) was tested in the present study. Isometric tension measurement of rat aorta and cytoplasmic Ca^{2+} determination in VSM cells (VSMC) were applied. NO was found to inhibit the KCl-induced Ca^{2+} entry in VSM. Moreover, NO was shown to selectively inhibit intracellular Ca^{2+} release induced by phenylephrine but not by caffeine in VSMC.

VSM contains NO synthase (NOS) system which can be induced to express after prolonged exposure to endotoxin and cytokines. There is little or no observable NOS activity in VSM under basal conditions (without stimulation by endotoxin and cytokines). We hypothesized that NOS activity in VSM under basal conditions might be masked probably due to its inhibition by protein kinases. H-7, a non-specific inhibitor of protein kinases, was used to remove this inhibition. Isometric tension measurement was performed on endothelium-removed rat aortic rings. In the presence of H-7, phenylephrine-induced concentration-contraction in denuded aortic rings was reduced in a dose-dependent manner. The inhibitory effect of H-7 could be greatly blocked by NOS inhibitors, which was totally reversed by L-arginine but not by D-arginine. The action of H-7 occurred rapidly and disappeared after washout of H-7. These data suggest that the inhibitory effect of H-7 is mediated by increased activity of existing NOS in VSM. Thus, NOS activity is masked in VSM under basal conditions probably due to its inhibition by protein kinases.

This study provides a new insight into the regulation of vascular contractility by NO in VSM.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Free calcium ions
$[Ca^{2+}]_i$	Cytoplasmic free calcium concentration
cDNA	Complementary deoxyribonucleic acid
Cyclic GMP	Guanosine 3', 5'-cyclic monophosphate
DMEM	Dubecco's Modified Eagle Medium
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N', N', N', N'-tetraacetic acid
ER	Endoplasmic reticulum
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FMN	Flavin mononucleotide
H-7	1-5(-isoquinosulfonyl)-2-methylpiperazine
Hb	Hemoglobin
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid

IL-1	Interleukin-1
IFN-γ	Interferon-γ
IP_3	Inositol 1, 4, 5-triphosphate
Kb	Kilobase
KD	Kilodalton
K_m	Michaelis constant
LHRH	Luteinizing hormone releasing hormone
L-NMMA	N^{ω}-monomethyl-L-arginine
L-NNA	N^{ω}-nitro-L-arginine
LP	Lipopolysaccharide
MB	Methylene blue
MEM	Minimal essential medium
NADPH	Nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
cNOS	Constitutive nitric oxide synthase
iNOS	Inducible nitric oxide synthase
PARS	Poly (ADP ribose) synthetase
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SHR	Spontaneously hypertensive rat
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
VSM	Vascular smooth muscle
VSMCs	Vascular smooth muscle cells

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CHAPTER I. INTRODUCTION

The discovery that mammalian cells produce nitric oxide (NO), a gas previously considered to be an atmospheric pollutant, is providing important information about many biological processes (Moncada et al., 1993). NO is synthesized from the amino acid L-arginine by nitric oxide synthase (NOS) in biological systems. NO is a free radical and has the lowest molecular weight of any known bioactive mammalian cell secretory product (Nathan, 1992). It acts as an intra- and transcellular signal in many biologic activities. During the past decade, intensive investigation has been performed on this novel mediator to elucidate its physiological and pathophysiological roles, biosynthetic mechanism and regulation, and therapeutic implications. The present review attempts to summarize all these aspects with emphasis on the cardiovascular system.

1. NO in Cardiovascular System

1.1. Observations of EDRF

In 1980, Furchgott and Zawadzki reported that the relaxation of rabbit aorta induced by acetylcholine (ACh) was dependent on the presence of the endothelium. By using sandwich preparations of rabbit aorta (an endothelium-free strip whose contraction is monitored, is laid intimal surface against intimal surface with an endothelium-containing strip), it has been shown that endothelium-dependent relaxation by ACh is mediated by a labile humoral factor (Furchgott and Zawadzki, 1980), later

known as endothelium-derived relaxing factor (EDRF) (Furchgott, 1983). Since then, endothelium-dependent relaxation has also been demonstrated in many vascular preparations, including some veins, arteries and microvessels in response to a variety of substances such as ACh, adenine nucleotides, thrombin, substance P, the calcium ionophore A23187 and bradykinin. Other stimuli, such as hypoxia, shear stress and electrical stimulation, also induce endothelium-dependent relaxation of vascular tissues *in vitro*. Other agents, however, such as nitrovasodilators, β-adrenergic agonists, and prostacyclin, cause vascular relaxation in an endothelium-independent manner (For reviews, see Griffith et al., 1984; Busse et al., 1985; Moncada et al., 1986).

It has been well established that EDRF is a very short-lived substance with a half-life of only seconds in oxygenated physiological salt solutions (Griffith et al., 1984; Cocks et al., 1985). EDRF is released under basal conditions as well as after stimulation with ACh (Griffith et al., 1984; Rubanyi et al., 1985; Martin et al., 1985). The effects of EDRF are mediated by intracellular accumulation of cyclic GMP following the activation of soluble guanylate cyclase, which is inhibited by hemoglobin (Hb), methylene blue (Martin et al., 1985) and other agents such as dithiothreitol and hydroquinone (Griffith et al., 1984). In addition, superoxide dismutase (SOD) is shown to prolong the effects of EDRF (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1984), indicating that superoxide anions (O_2^-) contribute to the instability of EDRF.

1.2. Identification of EDRF as NO

At a symposium in 1986, Furchgott proposed that EDRF might be NO. His proposal was based on the evidence that the transient relaxation of rabbit aorta induced by NO generated from additional NO_2^- is similar to that by EDRF (Furchgott, 1988). It has been found that NO is labile and capable of relaxing vascular preparations which can be blocked by hemoglobin and by generators of O_2^-, but remarkably potentiated by SOD. All of these properties are similar to those of EDRF. The strongest evidence for the identification of EDRF as NO comes from the experiments in which EDRF released from vascular endothelial cells is detected by the chemical means used to identify NO (Palmer et al., 1987). NO may be measured directly as the chemiluminescent product of its reaction with ozone (Downes et al., 1976). Using this method, it has been shown that the concentrations of bradykinin which induce the release of EDRF from porcine aortic endothelial cells in culture also cause a concentration-dependent release of NO. Moreover, the amount of NO released by bradykinin quantitatively accounts for the relaxation of the bioassay strip by the peptide. In addition, the levels of NO released by the endothelial cells also account for the inhibition of platelet aggregation and adhesion by EDRF (Radomski et al., 1987a, b) and their biological half-life as inhibitors of platelet aggregation are similar. The biological actions of both EDRF and NO on vascular strips and on platelets strongly support the proposal that EDRF is NO (Moncada et al., 1991).

Although the evidence for EDRF being NO is strong , controversial results have also been reported, including variations in the half-life of EDRF (from 3 to 50 s), different binding of EDRF and NO to anion exchange columns, and different effects of

EDRF and NO on smooth muscle preparations. The settlement of this question is dependent on the elucidation of the biochemical mechanism of NO synthesis.

The existence of other endothelium-dependent vasodilators is not surprising. They include endothelium-derived hyperpolarizing factor (EDHF), adenosine and prostacyclin (Feletou et al., 1988; Furchgott and Vanhoutte, 1989). It has been suggested (Moncada et al., 1991) that endothelium-dependent relaxation that is susceptible to inhibition by Hb and methylene blue indicates the involvement of NO and cyclic GMP in many vascular preparations whereas evidence for other mechanisms is insufficient. Once specific inhibitors of NOS (discussed in the following section) were found, their application greatly helped to determine whether NO is involved in biological activities.

1.3. Roles of Cyclic GMP in NO-mediated Vascular Relaxation

Before the discovery of endothelium-dependent vascular relaxation, it was known that nitrovasodilators activate soluble guanylate cyclase of vascular smooth muscle via the production of NO and that the following accumulation of cyclic GMP leads to the vascular relaxation. Several groups of investigators have demonstrated that endothelium-dependent relaxation is mediated by stimulating an increase in cyclic GMP levels (Rapoport et al., 1983; Diamond et al., 1983). A good correlation exists between the elevation of cyclic GMP and the degree of endothelium-dependent relaxation produced by ACh, A23187 and other agents. The increase in cyclic GMP precedes the relaxation, indicating a causal role for this cyclic nucleotide. Endothelium-dependent

relaxation is not accompanied by alteration in the level of cyclic AMP, and the increase in cyclic GMP occurs in the smooth muscle cells rather than in endothelial cells (Rapoport et al., 1983). EDRF has been shown to directly activate soluble guanylate cyclase (Förstermann et al., 1986b; Ignarro et al., 1986). Furthermore, it has been found that NO interacts with the ferrous iron in the heme prosthetic group of the soluble guanylate cyclase in vascular smooth muscle cells (VSMC), increasing the concentration of cyclic GMP which leads to the vascular relaxation (Waldman et al., 1988). Several mechanisms have been proposed to explain cyclic GMP-evoked relaxation, including inhibition of inositol triphosphate (IP_3) generation, stimulation of membrane Ca-ATPase, activation of Ca^{2+}-activated K^+ channels, and inhibition of Ca^{2+} channels. (Marín and Sánchez-Ferrer, 1990).

The NO-cyclic GMP pathway has been shown to represent a wide-spread signal-transduction system and is involved in the regulation of different biological processes, such as relaxation of vascular smooth muscle (Rapoport et al., 1983), inhibition of platelet aggregation (Mellion et al., 1981), and platelet adhesion to the vessel wall (Radomski et al., 1987b), as well as synaptic transmission in both the central and peripheral nervous systems (Garthwaite et al., 1989). These aspects will be discussed in the following sections.

1.4. Physiological Roles of NO

The identification of EDRF as NO led to the discovery of NOS in the vasculature (Palmer et al., 1988a) which generates NO from the amino acid L-arginine.

Furthermore, the methylated L-arginine analogue N^{ω}-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of NOS, provides a useful tool to investigate the roles of NO in biological processes. L-NMMA induces an endothelium-dependent constriction of rabbit aortic rings and inhibits endothelium-dependent relaxation produced by ACh, A23187 and substance P, which can be reversed by L-arginine (Palmer et al., 1988a; Rees et al., 1989a). In anesthetized rabbits, intravenous administration of L-NMMA, but not D-NMMA, causes an increase in blood pressure that is reversed by L-arginine but not D-arginine (Rees et al., 1989b). Moreover, it has been reported that L-NMMA induces a vasoconstriction of the forearm arterial circulation in humans (Moncada, 1992). In different vascular preparations including resistance vessels (Amezcua et al., 1989), microcirculation (Rees et al., 1990a) and many other preparations (Gardiner et al., 1990), L-NMMA also induces an endothelium-dependent vasoconstriction. These findings suggest that NO plays a physiologic role in regulating blood flow and pressure. The NO-dependent vasodilator tone seems to be maintained through the physical activation of endothelial cells by stimuli such as pulsatile flow and shear stress (Pohl et al., 1986; Rubanyi et al., 1986a).

NO is a mediator not only regulating vascular tone but also controlling myocardial contractility. It has been reported that a negative inotropic effect can be observed after the removal of the endo-cardium in isolated papillary muscle preparations (Smith et al., 1991). Schulz et al (1991) have found that cultured porcine endocardial cells release NO and possess a NO synthase. These results suggest that the L-arginine:NO pathway contributes to the regulation of myocardial contractility (Lewis et al., 1990).

Based on the observations that NO is generated extensively in the cardiovascular system both under basal conditions and upon stimulation, it has been suggested that NO can be considered an endogenous nitrovasodilator (Moncada et al., 1988). Without a doubt, the progress in the research of endogenous NO and of nitrovasodilators make contributions to each other. Inorganic nitrovasodilators (e.g. sodium nitroprusside, SNP) release NO spontaneously while organic nitrovasodilators (e.g. 3, morpholino-sydnonimine) liberate NO subsequent to base-catalysed hydrolysis (Feelisch and Noack, 1991). NO released by these nitrovasodilators in turn activates soluble guanylate cyclase and leads to the accumulation of cyclic GMP (Feelisch and Noack, 1987). The evidence strongly suggests that NO is the final common effector molecule of all nitrovasodilators that activate soluble guanylate cyclase (Moncada et al., 1991).

1.5. Pathophysiological Roles of NO

The concept that NO is an endogenous vasodilator may also be significant for the understanding of some pathophysiological observations. Impaired endothelium-dependent relaxation (Winquist et al., 1984) accompanied by reduced levels of cyclic GMP (Otsuka et al., 1988) has been reported in animal models of hypertension. The vasodilator response to ACh infusion into the brachial artery is reduced in patients with essential hypertension (Linder et al., 1990), while the response to SNP is not altered in such patients. Calver et al. (1992) has found that the response of forearm blood flow to L-NMMA is attenuated in patients with essential hypertension, suggesting that decreased generation of NO may contribute to their hypertension. Endothelium-

dependent relaxation is also damaged in pulmonary arteries isolated from patients undergoing heart-lung transplantation for end-stage chronic lung disease (Dinh-Xuan et al., 1991). Not surprisingly, inhaled NO gas reverses pulmonary hypertension (Pepke-Zeba et al., 1991) and protects against the adult respiratory distress syndrome (Rossaint et al., 1993). Although there is a strong correlation between impaired endothelium-dependent relaxation and hypertension, other workers have shown no change (Hagen and Webb, 1984) or an increase (Konish and Su, 1983) in endothelium-dependent relaxation in arteries from hypertensive animals. In addition, it is still unknown whether impaired endothelium-dependent relaxation is the cause of hypertension or subsequent to this pathological change. Further study is needed to elucidate their relationship.

Xiao and Pang (1994) reported that vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR) significantly inhibited the proliferation of lymphocytes from SHR and the genetic control, Wister-Kyoto rats (WKY). This inhibition was reversed by a NOS inhibitor, N^{ω}-monomethyl-L-arginine (L-NMMA). Upon stimulation, the VSMC from SHR produced an obviously higher amount of NO compared with those from WKY. Increased NOS activity in VSMC in SHR may be a compensatory reaction to the development of hypertension.

A decrease in EDRF release from vascular endothelium or a reduction in endothelium-dependent relaxation has been demonstrated in vascular tissues obtained from rabbits with atherosclerosis (Verbeuren et al., 1986) and in human atherosclerotic coronary arteries (Förstermann, 1986). Furthermore, vasodilatation induced by

increased blood flow or acetylcholine is also impaired in the coronary circulation of patients with atherosclerosis (Cox et al., 1989), smokers, and children with familial hypercholesterolemia (Celermajer et al., 1992). The administration of L-arginine can normalize this dysfunction in patients (Drexler et al., 1991; Creager et al., 1992).

It has been shown (Kanamaru et al.,1987) that endothelium-dependent relaxation induced by A23187 is inhibited by Hb and cerebrospinal fluid obtained from patients with subarachnoid hemorrhage. Moreover, intracisternal injections of Hb in the pig has been found to induce concentration-dependent vasoconstriction in intrathecal arteries (Byrne et al., 1989). These results indicate that scavenging of NO by Hb may play a role in the vasospasm that follows subarachnoid hemorrhage.

A calcium-independent isoform of NOS can be induced in vessel walls by certain cytokines and by the endotoxin lipopolysaccharide (LPS). The induction occurs in both endothelial and smooth muscle cells (Moncada and Higgs, 1993). This explains some observations in vascular preparations including the hyporesponses to vasoconstrictors (McKenna, 1990) and the elevations in cyclic GMP in smooth muscle after incubation with LPS (Fleming et al., 1990). These responses can be prevented by treatment with glucocorticoids and inhibitors of NOS. Thus, NO produced by this inducible enzyme accounts for the vasodilatation and resistance to vasoconstrictors in septic shock and the hypotension induced by cytokine therapy in patients with cancer (Moncada, 1992). Inhibitors of NOS have been used to treat patients and animals with hypotension resulting from septic shock.

It has been found that the concentrations of nitrite and nitrate, the oxidized metabolites of NO, are elevated in patients with cirrhosis and correlates with the degree of endotoxemia, suggesting an overproduction of NO generated by the inducible NOS (Guarner et al., 1993). These findings may therefore explain the hyperdynamic state of cirrhosis associated with vasodilatation and hyporesponsiveness to vasoconstrictors (Vallance and Moncada, 1991).

2. NO in Platelets

As early as 1981, NO was shown to inhibit platelet aggregation via a cyclic GMP-mediated mechanism (Mellion et al., 1981). Azuma et. al. (1986) found that EDRF also inhibits platelet aggregation. Radomiski et. al. (1987c) demonstrated that prostacyclin and NO act synergistically to inhibit platelet aggregation and to disaggregate platelets, indicating that the release of NO and prostacyclin by the vascular endothelium plays a role in its protecting the vascular wall against thrombus. The difference between NO and prostacyclin is that prostacyclin inhibits the aggregation of platelets by increasing the concentration of cyclic AMP (Radomski and Moncada, 1991). In addition, NO also inhibits platelet adhesion to collagen fibrils, endothelial cell matrix, and endothelial cell monolayers (Radomski et al., 1987b, d), which is also mediated by cyclic GMP. NO from endothelial cells or from exogenous nitrovasodilators inhibits platelet activation with a reduction in agonist-induced $[Ca^{2+}]_i$. Thus, myosin light chain phosphorylation in platelets is inhibited and subsequently platelet morphology is altered, reducing their

ability to aggregate (Lincoln and Cornwell, 1993). Furthermore, platelets themselves also generate NO when stimulated with collagen and the L-arginine:NO pathway may act as a negative feedback mechanism to inhibit platelet activation (Radomski et.al., 1990). The formation of NO from L-arginine in the platelet cytosol is dependent on the free Ca^{2+} concentration, showing that the NO synthesis in platelets is Ca^{2+} dependent. It is interesting that L-arginine does not increase the basal levels of cyclic GMP in unstimulated platelets but does so when they are stimulated with collagen. This shows that NO synthesis in platelets can utilize exogenous substrate after they are activated. Thus, platelet aggregation *in vivo* can be regulated by platelet-derived NO as well as by NO and prostacyclin released from vascular endothelium. Nitrovasodilators, combined with prostacyclin or its analogs, may therefore provide a useful antithrombotic therapy (Radomski and Moncada, 1991; Sinzinger et al., 1992).

3. NO in Nervous System

3.1. NO in the Central Nervous System

Garthwaite et. al. (1988) demonstrated that stimulation of rat cerebellar cells with N-methyl-D-aspartate (NMDA), an agonist for a specific type of glutamate receptor, releases an EDRF-like material, associated with an elevation of cyclic GMP levels. Moreover, the cells releasing this EDRF-like material in response to NMDA have been shown not to be the target cells in which cyclic GMP levels are increased. The increase in cyclic GMP levels has later been demonstrated to be enhanced by L-arginine and

inhibited by L-NMMA (Garthwaite et al., 1989), showing that this response is mediated by NO. These results together with the identification of a constitutive NOS in rat forebrain tissue (Knowles et al., 1989) have proven the existence of L-arginine:NO pathway in the central nervous system. NOS has now been detected in all areas of animal and human brains (Moncada and Higgs, 1993).

A. Physiological Roles of NO

More and more evidence indicates that NO may be a mediator of long-term potentiation (LTP) (O'Dell et al., 1991) and long-term depression (LTD) (Shibuki et al., 1991) of synaptic transmission. Both LTP and LTD are considered elements of experience-driven synaptic network remodeling that may underlie learning and memory. Thus, NO may play a role in the formation of memory. In *vitro*, after specific receptor stimulation, NO is released from a postsynaptic source to act presynaptically (retrograde action) on one or more neurons. This causes a further increase in the release of glutamate and as a result, a stable increase in synaptic transmission. This phenomenon is known as LTP. Moreover, inhibition of NOS in animal experiments impairs learning behavior, also suggesting the involvement of NO in memory (Chapman et al., 1992). A large population of NOS-containing neurons has also been found in hypothalamus. The effect of NO on the release of LHRH which is synthesized in hypothalamus has been investigated. By using arcuate nucleus-median eminance (MBH) explants from male rats *in vitro*, SNP, a NO donor, has been shown to increase the release of LHRH in a dose-dependent manner. L-NMMA has no effect on basal release of LHRH, but can completely block the norepinephrine-induced release of

LHRH. These results suggest that NO activates LHRH release. Cyclic GMP and prostaglandin E_2 have been shown to mediate this NO-induced LHRH release. LHRH controls mating behavior by actions in the brain and LHRH is also transmitted to the anterior lobe of pituitary where it causes release of gonadotropins that in turn stimulate the gonads to produce sex steroids (McCann and Rettori, 1996). In addition, NO may also play a physiological role in vision (Venturini et al., 1991), feeding behavior (Morley and Flood, 1991), nociception (Moore et al., 1991) and olfaction (Breer and Shepherd, 1993).

B. Pathophysiological Roles of NO

It has been known that excessive release of excitatory amino acids such as glutamate is associated with convulsion and neurotoxicity. The link between stimulation of receptors by these amino acids and activation of NOS leads to the suggestion that overproduction of NO may mediate the pathophysiological processes such as cerebral ischemia and epilepsy (Moncada et al., 1989). The primary stimulus for NO synthesis in the central nervous system is activation of NMDA-type glutamate receptors. Overactivity of NMDA receptors is implicated in numerous neurodegenerative processes including cerebral ischemia, stroke, Alzheimer's disease, Huntington's chorea, and amyotrophic lateral sclerosis (Lipton and Rosenberg, 1994). A causal role for NO in several models of NMDA-mediated degeneration is implied by neuroprotective effects of NOS inhibitors. Recent studies with NOS "knockout" mice also confirm that endogenous NO contributes to neurodegeneration (Huang et al., 1994). Consistent with this, NO donors mimic, and N^{ω}-substituted L-arginine analogs

block, neurotoxicity *in vitro* (Dawson et al., 1991). Therefore, inhibitors of NOS may be useful in the treatment of neurodegenerative diseases.

Microglial cells, which are considered macrophages in the central nervous system, can express the inducible NOS (Zielasek et al., 1992). These cells have been implicated in the pathogenesis of diseases such as multiple sclerosis, Alzheimer's disease, Parkinson's disease and the dementia of the acquired immunodeficiency syndrome (AIDS). Whether NO is associated with neuronal damage in these diseases remains to be elucidated.

3.2. NO in the Peripheral Nervous System

NO is also found in the peripheral nervous system, where it is released from many nerves previously classified as non-adrenergic, non-cholinergic (NANC) (Rand, 1992). In the gastrointestinal tract of rats, NO has been shown to mediate the dilation of the stomach to adapt to the increase of intragastric pressure (Rand, 1992). Histochemical studies of biopsy samples from infants with hypertrophic pyloric stenosis (Vanderwinden et al., 1992) and from adults with achalasia have demonstrated a lack of NOS in gastrointestinal tissue in these conditions (Mearin et al., 1993). Thus, a NO-dependent dilator tone may play an important role in the function of gastrointestinal tract.

Immunohistochemical studies have shown that nerves staining for NOS are present in penile tissue from different species including humans. Electrically stimulated relaxation of the corpus cavernosum can be prevented by NOS inhibitors (Ignarro et

al., 1990; Burnett et al., 1992) and mimicked by NO donors. Thus, NO is probably the final common mediator of penile erection and NO donors may be used to treat male impotency.

NOS is also found in the nerves of the urinary bladder (Persson et al., 1992), vasculature (Toda et al., 1992), and trachea (Belvisi et al., 1992). Therefore, a widespread system of nerves throughout the body may use NO as a neurotransmitter.

4. NO in Immunity and Inflammation

It has been found that macrophages synthesize nitrite and nitrate in response to LPS, IFN-γ, either alone or in combination with TNF (Stuehr and Marletta, 1987a, b; Ding et al., 1988). This generation is dependent on L-arginine and is responsible for the cytotoxicity of these cells against tumor cells and bacteria (Moncada and Higgs, 1993). Marletta and co-workers (1988) have showed that NO is the intermediate in the synthesis of oxides of nitrogen from L-arginine by macrophages.

The activation of macrophages by endotoxin and cytokines results in the induction of the Ca^{2+}-independent NOS which leads to the prolonged production of NO. NO diffuses to target cells such as tumor cells, bacteria, fungi, and helminths in which NO combines with iron-sulfur centers in key enzymes of both the respiratory cycle and the pathway for the synthesis of DNA (Moncada and Higgs, 1993) and inactivates these enzymes. The iron-sulfur containing enzymes include mitochondrial aconitase, succinate:ubiquinone reductase, and ribonucleotide reductase. The L-arginine:NO

pathway has been proposed to be the primary defense mechanism against intracellular microorganisms as well as pathogens such as fungi and helminths that are too large to be phagocytosed (Moncada, 1991). This non-specific immunity is a general phenomenon involving not only the reticuloendothelial system but also non-reticuloendothelial cells such as hepatocytes (Nussler et al., 1992), neutrophils (Moncada et al., 1992), vascular smooth muscle (Rees et all., 1990b) and vascular endothelium, in all of which the inducible NOS has been detected. Furthermore, NO has been shown to suppress allograft rejection, suggesting its involvement in specific immunity (Langrehr et al., 1991). However, the precise role is not yet clear.

Accumulating evidence indicates that NO may play a role in acute and chronic inflammation. Administration of inhibitors of NOS attenuates the severity of inflammation in rats with acute inflammation (Ialenti et al., 1992) or adjuvant arthritis (Ialenti et al., 1993), whereas L-arginine enhances it. These inhibitors also reduce immune complex-induced vascular injury in rat lungs and dermal vasculature (Mulligan et al., 1991). Moreover, the generation of NO in the colon is increased in patients with ulcerative colitis (Middleton et al., 1993) and inhibitors of NOS improve experimentally induced chronic ileitis (Miller et al., 1993), suggesting NO may be related to these chronic inflammatory diseases. Moreover, nitrite levels have been found to be elevated in plasma and synovial fluid in patients with rheumatoid arthritis and osteoarthritis (Farrell et al., 1992). The origin of NO in these situations is unclear, but it may come from blood vessels, neutrophils and macrophages.

Immune dysfunction including the suppressed lymphocyte proliferation response has been found in SHR. Xiao and Pang (1996) found that SHR spleen cell proliferation response was depressed in SHR at 4, 8, and 12 weeks and 1 year of age. This depressed response was corrected by either NOS inhibitor or removal of macrophages from spleen cells. NO production by SHR spleen macrophages was significantly high in all age groups, suggesting overproduction of NO may play a role in the suppressed lymphocyte proliferation responses in SHR.

Recent findings have demonstrated that overproduction of NO plays an important role in the formation of autoimmune diabetes which is characterized by an early insulitis or inflammation followed by the selective destruction of the pancreatic β cells (McDaniel et al., 1996). Recent studies indicate that cytokines (particularly IL-1) released from T lymphocytes and activated macrophages during this islet insulitis can induce iNOS expression by β cells and results in the overproduction of NO which may selectively destroy β cells (Corbett et al., 1994) by inactivating iron-sulfur-centered enzymes specifically located in the mitochondria of β cells. Moreover, cytokines may induce co-expression of, and NO produced by iNOS can directly activate, cyclooxygenase-2 (Cox-2), which is the rate-limiting enzyme responsible for the overproduction of the prostaglandins. These proinflammatory prostaglandins may be important in either initiating or maintaining the inflammatory response of autoimmune diabetes (McDaniel et al., 1996).

NO may also be associated with tissue damage, for it is cytostatic or cytotoxic not only for invading microorganisms but also for the cells that produce it and for

neighboring cells (Moncada, 1992). Both inhibitors of NOS (Buckberg et al., 1992) and NO donors (Lefer, 1992) have been reported to protect against some forms of injury. These results reveal the dual nature of NO, which is on the one hand cytotoxic and on the other cytostatic. The dual nature of NO suggests that the sensitivity to NO varies from one cell to another. The reasons for this are not yet clear, but they may be dependent on the relative importance of iron-sulphur-containing enzymes in different cells.

5. Biosynthesis of NO

5.1. Isoforms of NOS

Many mammalian cells are capable of synthesizing NO. Up to now, three isoforms of NOS (EC 1.14.13.39) have been identified as being responsible for this synthesis. Each isoform of NOS has been found in different tissues and on the other hand, the same tissue may express more than one isoform of NOS. More and more authors now prefer the nomenclature defined by Föstermann *et. al.* (1994) in which they have termed isoform I (in neuronal and epithelial cells), II (in cytokine-induced cells), and III (in endothelial cells) according to the order of their first purification and the isolation of their cDNAs. During the process of NO synthesis, a guanidino-nitrogen of L-arginine undergoes a five-electron oxidation, resulting in NO and the coproduct L-citrulline. Three isoforms of NOS are reviewed as follows.

A. Isoform I

Isoform I is constitutively expressed in brain and was first purified from rat and porcine cerebellum (Bredt and Snyder, 1990; Schmidt et al., 1991; Mayer et al., 1990). In the literature, this enzyme is also termed b-NOS (for brain NOS), c-NOS (for constitutive or Ca^{2+}-regulated NOS) or n-NOS (for neuronal NOS). Isoform I in brain is mostly a soluble enzyme that migrates with a molecular mass of 150 to 160 KD on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Some particulate activity can be detected in the presence of added FAD (Hiki et al., 1992) that shows the same immunochemical characteristics as the soluble form. Therefore, it is probably the same protein.

Purification of isoform I has permitted its molecular cloning from rat (Bredt et al., 1991) and human brain (Nakane et al., 1993). The deduced amino acid sequences predict proteins of 160 and 161 KD, respectively, which are consistent with the purification results. Moreover, the deduced sequences also show as high as 36% identity to cytochrome P450 (CPR) in its C-terminal half. The homology to CPR reflects the oxidative mechanism of NO biosynthesis. Isoform I contains the binding sites for required co-factors including NADPH, FAD, FMN, biopterin and calmodulin. Isoform I is highly conserved between species (93% identity between rat and human). The gene for the human isoform I is located to the 12q24.2→24.31 region of chromosome 12 as determined by the study of fluorescence in situ hybridization (Marsden et al., 1993; Xu et al., 1993b)

B. Isoform II

Isoform II is usually not constitutively expressed in cells but can be induced in macrophages and many other cells with bacterial endotoxin and/or cytokines (Stuehr et al., 1991; Hevel et al., 1991). Thus, this enzyme is also referred to as inducible NOS (iNOS). Isoform II is predominantly a soluble enzyme. The enzyme purified from induced mouse macrophages migrates with a molecular mass of 125 to 135 KD. Using brain NOS cDNA as a homologous probe (Lyons et al., 1992) and using macrophage NOS antibody for expression cloning (Xie et al., 1992), cDNA encoding isoform II has been cloned from mouse macrophages. Furthermore, it has also been cloned in rat smooth muscle (Nunokawa et al., 1993), rat liver (Adachi et al., 1993), rat and human hepatocytes (Wood et at., 1993; Geller et al., 1993) and a human colorectal adenocarcinoma cell line (Shermann et al., 1993). The cDNA of this enzyme predicts a protein of 130 to 131 KD molecular mass, which is in good accordance with data from the purified protein from murine macrophages. Increasing evidence shows as high as 97.8~99.1% identity between cDNA sequence of the enzyme from different cells in the same species (Förstermann et al., 1994), suggesting that NOS II in different cells of the same species represents the product of a single gene. Molecular study has revealed that the promoter region of the gene for macrophage-induced NOS shows a pattern for complex regulation (Lowenstein et al., 1993; Xie et al., 1993). There are two distinct regulatory regions upstream of the TATA box. The first region (position -48 to -209) is responsive to LPS, suggesting it mediates LPS-induced gene expression. The second region (position -913 to -1029) mediates the potentiation of the LPS-induced NOS activity by interferon gamma (IFN-γ), although IFN-γ alone cannot increase its activity.

The human gene for isoform II of NOS has been localized to the 17p11→17q11 region of chromosome 17 (Xu et al., 1993a).

C. Isoform III

Isoform III of NOS is constitutively expressed in endothelial cells. It is commonly called eNOS (endothelial NOS) or cNOS (constitutive NOS) in the literature. Unlike the two other isozymes, it is more than 90% particulate (Förstermann et al., 1991). On denaturing SDS-polyacrylamide gels, isoform III purified from native and cultured bovine aortic endothelial cells shows a molecular mass of 135 KD (Pollock et al., 1991). Using low-stringency screening strategies based on the DNA sequence of isoform I of NOS from brain, cDNA encoding isoform III has been cloned from bovine (Sessa et al., 1992) and human endothelial cells (Janssens et al., 1992). The cDNA clones predict proteins with a molecular mass of 133 KD for both species which is again consistent with that obtained from purified NOS for endothelial cells. The amino acid sequence of isoform III deduced from the cDNA shares 60% of homology to isoform I from brain. Moreover, the amino acid sequences of bovine and human NOS III isoforms are 94% identical. A unique feature of the isoform III is a consensus sequence for N-terminal myristoylation. This explains the particulate localization of the enzyme (Lamas et al., 1992). Site-directed mutagenesis of the myristoylation sequence from glycine to alanine converts isoform III to a 92% cytosolic enzyme (Sessa et al., 1993). Kinetic analysis of the wild-type and mutated enzymes has shown a similar K_m values for L-arginine (2 to 4 μmol/L), suggesting that the mutation may not affect the function of this isoform. The gene for the NOS III isoform from human endothelial

cells is a large gene, which contains 26 exons spaning 21 Kb and is localized to the 7q35-7q36 region of chromosome 7 (Marsden et al., 1993).

5.2. Distribution of NOS in Tissues

Among the three isoforms of NOS, isoform I is most widely distributed. Isoform I is expressed in almost all areas of the brain. Immunohistochemistry studies using specific antibodies to this enzyme (Schmidt et al., 1992) have demonstrated that it is also expressed in some areas of the spinal cord (Dun et al., 1992), in sympathetic nerves (Sheng et al., 1992), in epithelial cells of lung, uterus, stomach and in kidney macula densa cells (Schmidt et al., 1992). Interestingly, northern blot analysis has revealed that the mRNA of this enzyme is more abundant in human skeletal muscle than in human brain (Nakane et al., 1993). Biochemical assays of skeletal muscle homogenates indicate that over 90% of NOS activity is membrane associated and immunohistochemical localization of this enzyme shows prominant labeling of sarcolemma in skeletal muscle (Kobzik et al., 1994). Moreover, it has been suggested that endogenous NO produced in active muscle near the sarcolemma opposes contractile force (Bredt, 1996).

It has been known that many cell types can be induced to express NOS isoform II. In each case, its expression seems to be dependent on the exposure of cells or tissues to endotoxin and/or cytokines. Not many immunohistochemical studies have been reported so far. Isoform II of NOS has been shown to localize in macrophages; occasional lymphocytes; neutrophils, and eosinophils in red pulp of the spleen; Kupffer

cells; endothelial cells and hepatocytes in liver; alveolar macrophages in lung; macrophages and endothelial cells in adrenal glands, and histocytes, eosinophils, mast cells, and endothelial cells in the colon (Bandaletova et al., 1993). Isoform II is also expressed in vascular smooth muscle cells after exposure to cytokines (Koide et al., 1993).

Immunohistochemical studies using specific antibodies to isoform III have shown that this enzyme is relatively specific for endothelial cells (Pollock et al., 1993). It is found in various types of endothelial cells for arteries and veins in many tissues including human tissues.

5.3. Regulation of NOS

A. Physiological Regulation of NOS

Isoform I is a Ca^{2+}- and calmodulin-dependent enzyme that is inactive at 100 nmol/L Ca^{2+} and fully active at 500 nmol/L (Förstermann et al., 1990). In the brain, a stimulus such as glutamate acting at NMDA receptors triggers Ca^{2+} influx, which in turn binds to calmodulin, thereby activating NOS. The calmodulin binding regulates the electron transfer and oxygen activation activities of NOS, but does not affect arginine binding (Bredt and Snyder, 1994). The enzyme can be phosphorylated by Ca^{2+}-calmodulin-dependent protein kinase, protein kinase C (PKC), and cyclic AMP-dependent protein kinase (PKA) (Nakane et al., 1991; Bredt et al., 1992). Phosphorylation of NOS isoform I by PKC significantly attenuates the activity of this enzyme (Dawson et al., 1993).

It has been well established that isoform II of NOS is transcriptionally regulated by cytokines and endotoxin. These agents are termed inductants which activate NOS within hours in a manner sensitive to inhibitors of DNA transcription or mRNA translation. Once isoform II is induced, NO release can continue for days. What terminates high-output NO production is not yet known. The activity of isoform II is Ca^{2+}- and calmodulin-independent. However, this enzyme contains a binding site for calmodulin (Xie et al., 1992). Calmodulin is found to bind to the enzyme so tightly that it can be considered a subunit of the enzyme (Cho et al., 1992). In contrast, inducible NOS from human hepatocytes has been shown to lose its activity after calcium chelation and exposure to a calmodulin antagonist, suggesting a looser binding of calmodulin to this enzyme (Geller et al., 1993).

Isoform III is found constitutively expressed in endothelial cells. Like isoform I, it is Ca^{2+}-(100-500 nmol/L) and calmodulin-dependent (Pollock et al., 1991; Förstermann et al., 1991). Shear stress of blood flow not only increases endothelial NO release acutely (Lamontage et al., 1992) but also upregulates NOS isoform III expression (Nishida et al., 1992). A putative shear stress-responsive element has been found in the promoter sequence of NOS III (Marsden et al., 1993). In addition, this enzyme has been shown to undergo phosphorylation in response to bradykinin and after exposure to shear stress. The physiological importance of phosphorylation for regulating its activity is unknown.

B. Pharmacological Regulation of NOS

Pharmacological regulation of NOS relies on various inhibitors of NOS which can be classified into five categories. N^{ω}-substituted L-arginine analogs are the most commonly used inhibitors of all isoforms of NOS. They competitively block the activity of NOS, but this blockade is reversible in the presence of excess dose of L-arginine. These inhibitors are usually not specific for one isoform of NOS and have different potencies which may be due to the differences in uptake, distribution or metabolism of the compounds. Constitutive NOS from endothelial cells and inducible NOS from macrophages display different rank order of susceptibility to inhibition by various L-arginine analogs. N^{ω}-nitro-L-arginine (L-NNA) is the most potent known inhibitor (K_i = 200-500 nM) of the brain and endothelial constitutive NOS (isoform I and III, respectively), while L-N^{ω} aminoarginine (L-NAA) is the strongest inhibitor of the inducible NOS (isoform II) from macrophage (K_i = 1-5 μM) (Bredt and Snyder, 1994). It is expected that clinically useful inhibitors of NOS need to be isoform specific. For instance, potential neuroprotection by a NOS inhibitor may be mitigated by hypertension elicited by its inhibitory effect on endothelial constitutive NOS (Bredt and Snyder, 1994). So far, some NOS inhibitors have been found to be specific for one isoform of NOS. 7-Nitroindazole has been shown to preferentially influence brain NOS, although its K_i values for brain NOS and endothelial NOS are similar (Moore et al., 1993). In addition, aminoguanidine has been identified as a specific inhibitor for inducible NOS (isoform II) (Griffiths et al., 1993). It is predictable that development of specific inhibitors of NOS will provide new approaches to the treatment of diseases.

Another class of inhibitors are compounds that bind calmodulin and subsequently prevent its binding to target enzymes. Such compounds are specific for constitutive NOS (isoform I and III) which are calmodulin-dependent (Bredt and Snyder, 1990). One of these inhibitors, trifluperazine (IC_{50} = 10 μM) has been found to inhibit both endothelial and brain NOS (Bredt and Snyder, 1990). However, the ubiquitous existence and diverse functions of calmodulin obviously restrict the applications of this category of NOS inhibitors in the clinical therapy.

The third class of inhibitors are the covalent modifiers of NOS, represented by diphenylene iodonium and its congeners (Stuehr et al., 1991). The aromatic iodoniums are extremely potent. The inhibitory concentrations are about two orders of magnitude lower than for N^{ω}-substituted L-arginine analogs. These inhibitors seem to compete with a nucleotide cofactor for binding to NOS since the onset of their inhibitory effect can be reversed by high concentrations of FAD, FMN, NADPH or certain of their structure constituents in a highly specific manner (Stuehr et al., 1991). The evidence that these agents also inhibit other flavoenzymes make them unlikely to be suitable for therapeutic purposes.

Carbon monoxide, which binds to the heme in NOS, can incompletely inhibit the activity of NOS (White and Marletta, 1992). The usefulness of heme-target inhibitors of NOS has yet to be described.

The fifth class of NOS inhibitors is represented by a substituted pyrimidine that interferes with the transformation of GTP to tetrahydrobiopterin (Werner-Felmayer et al., 1990) which is another cofactor of NOS. Because tetrahydrobiopterin is required

for the synthesis of neurotransmitters, and because inhibition of NOS by depletion of tetrahydrobiopterin is likely to be slow in onset, the therapeutic potential of inhibitors of this class seems to be limited.

6. Fate of NO in the Body

6.1. Metabolism of NO in the Body

NO is a colorless gas that dissolves in water in the absence of oxygen. In air, NO reacts rapidly with oxygen to form nitrogen dioxide, a brown gas capable of causing tissue damage. In water and plasma, NO is oxidized to nitrite, which is stable for several hours. In animal blood, NO is found mostly bound to albumin (Stamler, 1994). This finding is interesting because NO in the nitrosothiol form has a much longer half-life and is potentially delivered to spatially or temporally distant targets. In humans, ingested ^{15}N-labeled L-arginine is excreted in part as [^{15}N] nitrate in the urine (Leaf et al., 1990). NO is also rapidly oxidized to higher oxides of nitrogen and as a result, nitrosates molecules containing sulfhydryl groups, such as glutathione, cysteine, and albumin. The kinetics of the association and dissociation of these molecules, retaining or yielding NO, may vary such that some of them may act as carriers or as biologic sinks for NO (Stamler et al., 1992). It seems that the fate of NO in the body is complex. However, a major metabolic transformation of NO when given by inhalation is by means of hemoglobin into nitrate (Yoshida et al., 1980).

6.2. Molecular targets of NO

Accumulating evidence has revealed that NO can act at various molecular targets to influence many biological activities. NO can interact with almost all cell components, including proteins, carbohydrates, lipids, and nucleic acids and various small molecules (McDonald and Murad, 1996).

Most interactions of NO with enzymes cause inactivation with the notable exception of guanylate cyclase. NO has been shown to activate soluble guanylate cyclase by binding to the iron in the heme, which is at the active site of the enzyme, altering the enzyme's conformation to augment catalysis, leading to an accumulation of cyclic GMP. This has been proven to be a cardinal mechanism of action of NO in vascular smooth muscle, as NO-induced vasodilatation can be mimicked by membrane-permeant forms of cyclic GMP, blocked by inhibitors of soluble guanylate cyclase, and augmented by inhibitors of cyclic GMP phosphodiesterase (Ignarro,1990; Moncada et al., 1991). In addition, NO also binds to other heme-containing proteins, including hemoglobin and myoglobin. These reactions are the basis of several assays for NO production. The turning pink of myoglobin upon binding with NO is one reason why nitrites are used to cure meat (Ignarro, 1990).

NO can also bind to nonheme iron in numerous proteins, such as NADH-ubiquitous oxidoreductase, NADH:succinate oxidoreductase, and cis-aconitase, all iron-sulfur-containing enzymes (Nathan, 1992). NO binds to the iron in ferrin, an iron-storage protein, liberating the Fe and the free Fe may cause lipid peroxidation (Reif and Simmons, 1990). NO from macrophages is also shown to bleach the chromophore of

clostridial ferredoxin, another iron-sulphur protein, reflecting a mechanism of macrophage antimicrobial function (Stuehr and Nathan, 1989). The binding of NO to the nonheme iron of ribonucleotide reductase has been found to inhibit DNA synthesis (Lepoivre et al., 1990). Ribonucleotide reductase is the rate-limiting enzyme of DNA synthesis. Its inhibition by NO accounts for the anti-tumor action of macrophage-derived NO (Nathan, 1992).

Another important target of biologically derived NO may be superoxide anions. The reaction of superoxide with NO may cause detoxification of both molecules or generate peroxynitrite (Beckman et al., 1990). The peroxynitrite may decay to nitrogen dioxide and hydroxyl radical which is considered the strongest oxide in biological systems.

In addition, NO itself or NO-generating compounds can be oxidized to a mutagen that can damage DNA by base deamination (Wink et al., 1991). This pathologic mechanism plays a key role in NO-induced neurotoxicity (Bredt and Snyder, 1994). DNA damage by NO stimulates the activity of poly (ADP ribose) synthetase (PARS). Massive activation of PARS depletes the cell of NAD and ATP. Thus the depletion of energy ultimately leads to the cell death. NO and its oxidation products peroxynitrite (and peoxynitrous acid) can also damage other cell constituents: lipid can be oxidized and peroxidized, and proteins can be oxidized at any of several groups (Stamler et al., 1992; Stamler, 1994).

NO is an important signal transduction mediator involved in autocrine, paracrine, and even in endocrine systems. Alteration in its production or activation contributes to pathological states, some of which have been discussed in this review. With the

progress in NO research, it is predictable that other such conditions will soon be recognized.

Since NO is both cytostatic and cytotoxic, homeostasis of NO production and NOS activity is a critical issue in the body. Both NO donors and NOS inhibitors may be useful for clinical therapy. Up to now, the well known example is that inhaled NO is proving beneficial in the treatment of pulmonary hypertension and both infant and adult respiratory distress syndrome. The general distribution of NOS in different tissues implies that the future therapeutic application of NOS inhibitors need to be specific for one isoform to avoid side effects.

7. $[Ca^{2+}]_i$ and Smooth Muscle Contraction

7.1. Role of Ca^{2+}

It has been well known that Ca^{2+} is a central second messenger in smooth muscle contraction. In the past decade, identification of the sources of Ca^{2+} and the mechanisms involved in regulating the movement of Ca^{2+} across biological membranes have been extensively investigated. Since the discovery of the photoprotein, aequorin (Morgan and Morgan, 1982), and fluorescent Ca^{2+} indicator dyes, such as quin-2, fura-2, indo-1, it has been well established that the $[Ca^{2+}]_i$ in resting smooth muscle cells is within the range of 120-270 nM (DeFeo and Morgan, 1985; Williams and Fay, 1986; Williams et al., 1987). The VSMC which exhibit intrinsic resting tone have higher

$[Ca^{2+}]_i$. Upon stimulation, the free $[Ca^{2+}]_i$ increases rapidly and transiently, usually averages from 500-700 nM (Williams and Fay, 1986).

7.2. Regulation of $[Ca^{2+}]_i$ in VSM

$[Ca^{2+}]_i$ in vascular smooth muscle increases during contraction either as a consequence of Ca^{2+} influx from the extracellular space or release of Ca^{2+} from intracellular stores.

A. Ca^{2+} Entry through the Plasma Membrane

Ca^{2+} influx across the plasma membrane under physiological conditions occurs through ion channels. Ion channels on the plasma membrane responsible for Ca^{2+} entry can be classified as voltage-dependent and voltage-independent. Up to now, T- and L-type channels (transient and long lasting, respectively) have been identified in smooth muscle cells (McDonald et al., 1994). The L-type channels are by far the best understood Ca^{2+} channels. These channels can be activated at high voltage, and are sensitive to dihydropyridine and other organic or inorganic blockers. L-type channels are responsible for the upstroke of the action potential and the influx of Ca^{2+} activated by membrane depolarization (Missiaen et al., 1992). Much less is known about T-type than about L-type Ca^{2+} channels.

Voltage-independent Ca^{2+} channels have been identified in several smooth muscle types. Some can be activated in response to specific receptor activation. The receptor-activated channel is usually non-selected (Horowitz et al., 1996).

B. Ca^{2+} Release from Internal Stores

Another way to raise cytosolic $[Ca^{2+}]_i$ is to stimulate Ca^{2+} release from internal stores. A large body of evidence suggests that the endoplasmic reticulum (ER) is the major Ca^{2+} store regulating cytoplasmic Ca^{2+} in smooth muscle while mitochondria are not (Somlyo and Himpens, 1989). At the level of the ER, there are two types of Ca^{2+} release channels. One is the inositol 1,4,5-triphosphate (IP_3) receptor. A variety of vasoactive substances can increase the level of IP_3 which is a soluble hydrolysis product of phospholipase C, leading to the Ca^{2+} release from the ER. The other is ryanodine receptor (Missiaen et al., 1992). Ryanodine receptors can be activated by Ca^{2+}, ATP, caffeine, and partially by ryanodine (Smith et al., 1985). However, the ability of Ca^{2+} to activate the smooth muscle ryanodine receptor under physiological conditions is still controversial. Although the ryanodine receptor shares features with the IP_3 receptor, such as a homotetrameric structure, it is not activated by IP_3 (Horowitz et al., 1996).

In contrary to elevation of $[Ca^{2+}]_i$, a decrease in $[Ca^{2+}]_i$ can be realized by restricting Ca^{2+} entry through the plasma membrane and by increasing active Ca^{2+} uptake into the ER or active Ca^{2+} extrusion across the plasma membrane.

7.3. Signaling Pathways

As mentioned above, cytoplasmic free Ca^{2+} level ($[Ca^{2+}]_i$) in smooth muscle is a major determinant of smooth muscle contractility. One consequence of increases in $[Ca^{2+}]_i$ levels in the smooth muscle cell is the activation of Ca^{2+}/calmodulin-dependent

myosin light kinase (MLCK) and phosphorylation of the myosin light chain, resulting in an increase in myosin ATPase activity and, subsequently, cross-bridge cycling.

Agonist-induced contraction of vascular smooth muscle usually involves both a rapid phasic and a sustained tonic component. Activation of α_1-adrenoreceptor in vascular smooth muscle by phenylephrine, induces intracellular Ca^{2+} release via IP_3 pathway leading to phasic contraction in Ca^{2+}-free medium followed by a sustained tonic contraction when extracellular Ca^{2+} is present. On the other hand, KCl activates vascular smooth muscle by membrane depolarization that initiates Ca^{2+} influx through voltage-gated Ca^{2+} channels, leading to a sustained contraction.

Although elevation of $[Ca^{2+}]_i$ and myosin light chain phosphorylation may account for agonist-induced tonic contraction, sustained tension developed may also occur without detectable sustained elevation of $[Ca^{2+}]_i$ and myosin light chain phosphorylation (Aksoy et al., 1983; Horowitz et al., 1996). Protein kinase C and rearrangement of intermediate filaments have been shown to be involved in such sustained contraction in vascular smooth muscle (Rasmussen et al., 1987).

8. Overall Hypothesis

Although it has been established that NO reduces vascular contractility in different vascular preparations, the precise mechanisms involved have not been well understood. Since $[Ca^{2+}]_i$ plays an important role in the regulation of vascular contractility, NO may impair vascular contractility by reducing $[Ca^{2+}]_i$ in VSM. In the present study, how NO

affects Ca^{2+} entry and intracellular Ca^{2+} release in VSM has been investigated with emphasis on the latter.

NO, no matter where it derives from, can only influence smooth muscle contraction following its diffusion to VSMC. Therefore, NO produced in VSM itself may also play a role in the regulation of vascular contractility. It has been well established that NOS in VSM can be induced to express after prolonged exposure to endotoxin and/or cytokines. There is little or no observable NOS activity in VSM under basal conditions (without stimulation with cytokines and/or endotoxin). It has been found that NOS activity can be regulated by different protein kinases and this may lead to its inhibition in gastric smooth muscle cells. Thus, it is hypothesized that NOS activity in VSM under basal conditions is probably inhibited by protein kinases.

9. Specific Aims

The present study attempts to achieve two aims as follows.

9.1. To study the effects of NO (from endothelium or NO donor) on agonist-induced Ca^{2+} entry and intracellular Ca^{2+} release in VSM on both tissue and cell levels. KCl was applied to stimulate Ca^{2+} entry in VSM in Ca^{2+}-containing solution while phenylephrine and caffeine were used to stimulate intracellular Ca^{2+} release through different pathways in VSM in a Ca^{2+}-free environment.

9.2. By using H-7, a non-specific inhibitor of protein kinases, to remove the assumed inhibition of NOS activity in VSM under basal conditions, it was

investigated whether the inhibitory effect of H-7 on the phenylephrine-induced concentration-contraction in endothelium-removed aortic rings was mediated by increased activity of NOS in VSM.

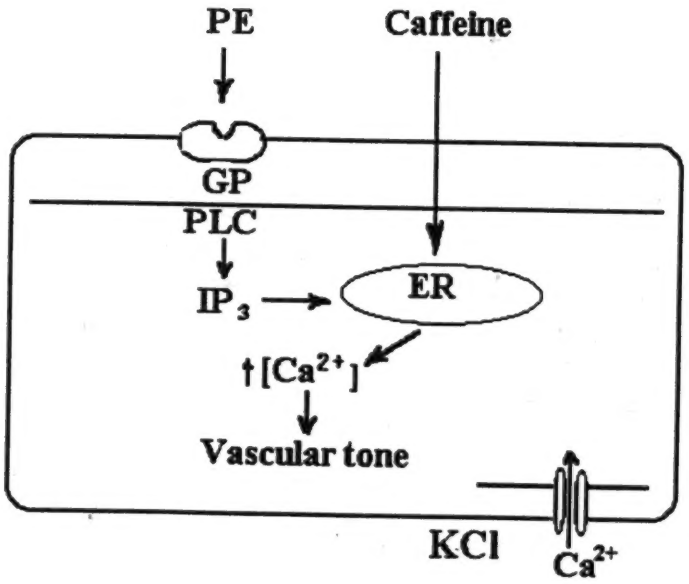

Fig.1-1. Schematic graph showing the elevation of $[Ca^{2+}]_i$ in VSMC induced by different agents. PE, phenylephrine. GP, G-protein. PLC, phospholipase C. IP_3, inositol-1,4,5-triphosphate. In the next section, it was investigated whether NO could affect each of these pathways to regulate vascular contractility.

CHAPTER II. MATERIALS AND METHODS

1. Isometric Tension Measurement

1.1. Vessel Preparations

After anesthetizing male Sprague-Dawley rats (250-300 g) with sodium pentobarbital (65 mg/kg, ip), each thoracic aorta was quickly removed and placed in cold Krebs solution of the following composition (mM, pH: 7.4): 115.0 NaCl, 5.0 KCl, 25.0 $NaHCO_3$, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 2.1 $CaCl_2$, 11.0 glucose. Fat and connective tissues surrounding the aorta were carefully removed. Then it was cut into vascular rings (2.0-2.5 mm in length). The endothelium was removed from some rings when necessary by gently inserting a cotton thread through the lumen of the rings. The aortic rings were then mounted in 10-ml water-jacketed bath organ chambers containing Krebs buffer at 37°C, gassed with 95% O_2 and 5% CO_2 bubbled through (Shan, 1993). For some aortic rings, they were preincubated with or without lipopolysaccharide in a tissue incubator for 6 hrs before mounted in the organ chambers. Isometric tensions generated by the arterial tissues were measured with a Grass FT.03 force displacement transducer and recorded on a Gould model serial polygraph (Gould Inc. Cleveland, Ohio.). The aortic rings were equilibrated at an initial resting tension of 1.0 g, and the buffer solution was changed every 15 min to stabilize the arterial tissues. The removal of the endothelium was functionally verified by the lack of a relaxant response to ACh (10^{-5} M) in aortic rings precontracted with phenylephrine (10^{-6} M). If not particularly indicated, the Krebs solution is Ca^{2+}-containing solution.

1.2. Single Dose Contractile Response Evoked by KCl

KCl is known to elicit the contractions of vascular smooth muscle through the depolarization of the cell membrane, which in turn activates Ca^{2+} channels (Hurwitz et al., 1980) followed by Ca^{2+} influx. KCl (30 mM) was applied to develop a sustained contraction. After the maximal tension was reached, the tissues were washed three times with Ca^{2+}-containing solution. The next administration of KCl was given after a 15 min interval. Data were collected when repeated responses appeared. ACh was used to stimulate the NO release from the endothelium. Since the half life of NO is only several seconds, ACh (10^{-5} M) was applied 5 sec before KCl was added when applicable.

1.3. Phasic Contractions Induced by Agonists in Ca^{2+}-free Solution.

Ca^{2+}-free Krebs solution was prepared by the omission of $CaCl_2$ and the addition of 0.2 mM EGTA. Before data collection, all aortic rings were contracted by phenylephrine or caffeine in Ca^{2+}-free solution for 5 min. Since the second application of the agonists was almost ineffective, the arterial tissues were then washed with Ca^{2+}-containing Krebs solution three times and incubated with it for 15 min (Ca^{2+} loading time). Subsequently, the medium was replaced by Ca^{2+}-free solution and 5 min later phenylephrine (10^{-6} M) or caffeine (20 mM) was applied to produce a phasic contraction. Only those tissues exhibiting repeated contractile responses of similar magnitude in Ca^{2+}-free solution were selected. In all experiments, N^{ω}-nitro-L-arginine (L-NNA), L-arginine, D-arginine, sodium nitroprusside (SNP), and methylene blue

were added to the organ chambers at the beginning of incubation period with Ca^{2+}-free solution. ACh (10^{-5} M) was added 5 sec before the administration of phenylephrine or caffeine.

1.4. Cumulative Concentration-response Evoked by Phenylephrine

In order to show inducible NOS activity in VSM, endothelium-denuded aortic rings were incubated for 6 hrs in 2.0 ml of minimal essential medium (MEM, Gibco) containing LPS (0, 3, 30, or 300 ng/ml). LPS was from Escherichia Coli and its serotype was 055:B5 (Sigma Chemical Co.) This incubation was performed in an tissue incubator with 5% CO_2 and 95% air at 37°C. Then, the aortic rings were mounted in the organ bath chambers containing the Krebs solution and washed three times with it. After the arterial tissues were stable, a serial dose-dependent contractions induced by phenylephrine (10^{-9} to 10^{-5} M) were obtained. The interval between the consecutive two concentration-responses was 30 min. L-NNA (10^{-5} M) or L-NNA plus L-arginine (10^{-4} M) was added during the interval when applicable.

In another set of experiments, aortic rings without endothelium were mounted in organ chambers without pretreatment with LPS. After the arterial tissues were stable, serial concentration-responses induced by phenylephrine (10^{-9} to 10^{-5} M) were recorded. The interval between the two adjacent concentration-responses was 30 min. After each response, the aortic rings were washed with Krebs solution at least 3 times and the basal tension were kapt at 1.0 g. H-7, L-NNA, aminoguanidine, L-arginine and D-

arginine (10^{-4} M) were added during the interval when applicable. One exception was methylene blue (10^{-5} M) which was added during last 10 min of the interval.

2. Isolation and Culture of VSMC

The method was previously described by Clapper and Gurney (1991) with some modification. Aortas were collected as described above. After the thoracic aorta was quickly taken out, it was immersed in cold physiological solution with the following composition (mM, pH: 7.4): 1.8 $CaCl_2$, 112.0 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 5.0 HEPES, 15.0 $NaHCO_3$, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 11.0 glucose, 0.04 phenol red. The connective tissues and adventitia were carefully removed and the cleaned vessel was opened along its longitudinal axis. About fifteen strips (~ 2 mm in width) were cut transversely and washed twice in the dissociation medium (DM) which contained (mM, pH 7.0): 0.16 $CaCl_2$, 110.0 NaCl, 5.0 KCl, 2.0 $MgCl_2$, 10.0 HEPES, 10.0 $NaHCO_3$, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 10.0 glucose, 0.04 phenol red, 0.49 EDTA, 10.0 taurine. They were then put into a separate dish containing 5 ml of DM with 0.1% bovine serum albumin in which 3.5 mg papain (17 units/mg protein; Sigma Chemical Co.) was dissolved. The arterial strips were stored overnight (~16 hrs) in this enzyme solution at 5-7° C. The next morning, dithiothreitol (0.1 mM, Sigma Chemical Co.) was added to the dish, which was then incubated at 37° C for about 12 min. The arterial strips were then transferred to fresh DM. Gentle trituration with a fire-polished Pasteur pipette for 3 min yielded long, relaxed, single vascular smooth muscle cells (VSMC). The viability

of the cells was tested with trypan blue exclusion. In our preparation , >85% of the cells did not take up the dye. Primary culture of VSMC was performed for the purpose of cytoplasmic Ca^{2+} concentration determination. The cells were seeded on a coverslip (22 mm circle, Fisher Scientific Co.) which had been placed in a 35 mm culture dish. Then, 2 ml Dulbecco's modified Eagle medium (DMEM, Gibco Life Technologies Inc., Grand Island, NY) with 10% fetal bovine serum (FBS, Sigma Chemical Co.) and 1% PSN antibiotic mixture (containing penicillin 5 mg/ml, streptomycin 5 mg/ml and neomycin 10 mg/ml, Gibco Life Technologies Inc., Grand Island, NY) was added to the dish. The cells were grown in DMEM supplemented with 10% FBS and the medium was changed every 48 hrs. The cells were kept in an atmosphere of 5% CO_2 and 95% air at 37°C. Confluence was obtained about 7-9 days after harvest when myocytes exhibited hills and valleys which are characteristics of confluent cultured VSMC.

3. Cytoplasmic Ca^{2+} Concentration ($[Ca^{2+}]_i$) Determination

$[Ca^{2+}]_i$ in VSMCs was determined according to the method described by Grynkiewicz *et al.* (1995) with some modification. Once the cells had reached confluence, they were incubated for 45 min in DMEM containing 5 μM fura-2 acetoxymethyl ester (fura-2/AM, Molecular Probes, Eugene, OR) in a dark compartment at room temperature. Subsequently, the cells were gently washed three times with 5K buffer solution of the following composition (mM, pH: 7.4):145.0

NaCl, 5.0 KCl, 1.0 $MgCl_2$, 10.0 glucose, 1.0 $CaCl_2$, 0.5 NaH_2PO_4, 10.0 HEPES. The coverslip with attached cells was placed in a 1-ml Sykes-Moore chamber on the stage of an inverted microscope (Diaphot-TMD, Nikon, Tokyo, Japan) and fluorescence measurement was made with a spectrofluorometer (Spex. Inc., Edison, NJ). $[Ca^{2+}]_i$ was calculated using the following formula: $[Ca^{2+}]_i$ (nM)=$K_d \times (R-R_{min})/(R_{max}-R) \times b$ where R was the fluorescence ratio of fura-2 excited at 340 nm and 380 nm and recorded at 510 nm (F340/F380). R_{max} and R_{min} were the fluorescence ratio determined by adding 2 μM ionomycin and subsequently adding 5 mM EGTA, respectively. b was the ratio of fluorescence of fura-2 when excited at 380 nm in the zero and saturating Ca^{2+}. K_d was the dissociation constant of fura-2 for Ca^{2+}, assumed to be 224 nM.

3.1. Measurement of $[Ca^{2+}]_i$ in VSMCs Using KCl as a Stimulator

Using KCl (15 mM) as a stimulator, $[Ca^{2+}]_i$ in VSMCs was determined in Ca^{2+}-containing 5K solution. After each response, the cells were washed three times with the same solution. The interval between the consecutive responses was 10 min. Data were collected from the second response. The application of SNP (10^{-7} M) was 5 min before KCl was added.

3.2. Measurement of $[Ca^{2+}]_i$ in VSMCs Using Phenylephrine as a Stimulator

In order to observe intracellular Ca^{2+} release, $[Ca^{2+}]_i$ was measured using phenylephrine (10^{-6} M) as a stimulator in the absence of extracellular Ca^{2+}. The cells were washed three times with Ca^{2+}-free 5K solution made of 5K solution with omitted

Ca^{2+} and added 0.2 mM EGTA, and incubated with this solution for 5 min. Then, phenylephrine (10^{-6} M) was added. After each response, the cells were washed with Ca^{2+}-containing 5K solution and incubated with it for 5 min (Ca^{2+} loading) followed by the replacement of Ca^{2+}- free 5K solution. Data were collected from the second response.

4. List of Chemical Substances and Their Sources

ACh	Sigma Chemical Co. Louis, MO, USA
Aminoguanidine	Sigma Chemical Co. Louis, MO, USA
BSA	Sigma Chemical Co. Louis, MO, USA
Dithiothreitol	Sigma Chemical Co. Louis, MO, USA
DMEM	Gibco Life Technologies Inc., Grand Island, NJ, USA
DMSO	Fisher Scientific Co., Fair Lawn, NJ, USA
EDTA	Sigma Chemical Co. Louis, MO, USA
EGTA	Sigma Chemical Co. Louis, MO, USA
FBS	Sigma Chemical Co. Louis, MO, USA
Fura-2/AM	Molecular Probes Inc. Eugene, OR, USA
H-7	Sigma Chemical Co. Louis, MO, USA
HEPES	Fisher Scientific Co., Fair Lawn, NJ, USA
Ionomycin	Sigma Chemical Co. Louis, MO, USA
L-NNA	Sigma Chemical Co. Louis, MO, USA

MEM	Gibco Life Technologies Inc., Grand Island, NJ, USA
Papain	Sigma Chemical Co. Louis, MO, USA
Pentobarbital sodium	M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada
Phenylephrine	Sigma Chemical Co. Louis, MO, USA
SNP	Fisher Scientific Co., Fair Lawn, NJ, USA
Taurine	Sigma Chemical Co. Louis, MO, USA

5. Statistics

Results of experiments were expressed as mean±S.E.M.. For analysis of concentration-contraction curves, the median effective concentration (EC_{50}) was manually calculated. The data were analyzed statistically with the Newman-Keuls test (analysis of variance, PHARM/PCS, version 4.2) among three or more groups or with student t-test between two groups. $P<0.05$ was accepted as statistically significant.

CHAPTER III. EFFECTS OF NO ON $[Ca^{2+}]_i$ IN VSM

1. Introduction

Regulation of vascular smooth muscle (VSM) tone by endothelial cells of large vessels has been ascribed to the release of vasoconstrictive and vasodilatory mediators (Furchgott, 1983). One of these mediators is endothelium-derived relaxing factor (EDRF) which has been identified as nitric oxide (NO; Moncada et al., 1991). Release of NO from vascular endothelial cells has been observed under basal conditions as well as after stimulation with a variety of substances such as acetylcholine (ACh), bradykinin, ATP and histamine (Moncada et al., 1991).

NO derived from vascular endothelial cells can easily diffuse to the underlying vascular smooth muscle cells (VSMC) where it activates soluble guanylate cyclase and leads to the accumulation of guanosine 3', 5'-cyclic monophosphate (cyclic GMP). Cyclic GMP is believed to cause vascular relaxation (Furchgott and Vanhoutte, 1989). Although the precise mechanisms by which NO:cyclic GMP causes vascular smooth muscle (VSM) relaxation are not well understood, several mechanisms have been proposed including dephosphorylation of the myosin light chain (Murad, 1986), interaction with the contractile apparatus (Karaki et al., 1988) and a reduction of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) (McDaniel et al., 1992).

A decrease in $[Ca^{2+}]_i$ can be realized through different ways including inhibition of Ca^{2+} entry across the plasma membrane and inhibition of intracellular Ca^{2+} release. It has been shown that NO has an inhibitory effect on Ca^{2+} entry through voltage-gated

calcium channels in VSMC (Blatter and Wier, 1994) and in cardiac myocytes (Levi et al., 1994). However, inconsistent results have been reported about how NO affects intracellular Ca^{2+} release. The vascular relaxation induced by EDRF has been shown to be partially due to the reduction of intracellular calcium release (Collins et al., 1986). In contrast, NO has been found not to influence [Arg^8]-vasopressin-induced intracellular Ca^{2+} release in cultured VSMC (Blatter and Wier, 1994). In other preparations such as interstitial cells in the mammalian gut (Publicover et al., 1993), pancreatic β cells (Willmott et al., 1995), and sea urchin eggs (Willmott et al., 1996), treatment with NO and NO donors has been demonstrated to mobilize intracellular Ca^{2+} release. Strict comparison among these experiments is difficult since different preparations and different agents have been used.

It has been known that the endoplasmic reticulum (ER) is the major intracellular Ca^{2+} store which plays an important role in regulating contractility of smooth muscle (Somlyo and Himpens, 1989). Two kinds of receptors (or channels) have been found on the membrane of the ER to regulate Ca^{2+} release (Missiaen et al., 1992). One is the inositol 1,4,5-triphosphate (IP_3) receptor that is sensitive to the elevation in cytosolic IP_3 concentration. The other is the ryanodine receptor which is directly activated by caffeine. Phenylephrine, an α_1-adrenoreceptor agonist, and caffeine can stimulate intracellular Ca^{2+} release through the IP_3 receptor pathway (Rembold, 1992) and the ryanodine receptor pathway (Missiaen et al., 1992), respectively. According to previous studies (Karaki et al., 1979; Karaki et al., 1988; Horie et al., 1991), phasic contractions generated in aortic rings by agonists in Ca^{2+}-free solution are the result of

Ca^{2+} release from internal stores of VSM. Furthermore, fluorescence measurement of fura-2, a Ca^{2+} indicator, provides a direct method to investigate the mobilization of internal Ca^{2+} stores in primary cultured VSMC isolated from rat aorta. Therefore, this study has been designed to investigate whether NO may interfere with intracellular Ca^{2+} release through different pathways. In addition, whether NO may affect Ca^{2+} entry through the plasma membrane was also investigated. Since KCl is known to contract VSM through the depolarization of the plasma membrane, which in turn activates calcium channels (Hurwitz et al., 1980), KCl-induced contraction was used to induce Ca^{2+} entry through the plasma membrane of VSM.

2. Experimental Design

2.1. Effect of NO on Ca^{2+} Entry.

Tension generation in intact aortic rings induced by KCl (30 mM) was used to stimulate Ca^{2+} entry through the plasma membrane of VSM (Hurwita et al., 1980). ACh (10^{-5} M) was applied to stimulate the release of NO from the endothelium. Since the half-life of NO was only several seconds, the addition of ACh was 5 sec before the administration of KCl.

In fura-2 study, $[Ca^{2+}]_i$ in primarily cultured VSMC was measured. KCl (15 mM) was applied to increase $[Ca^{2+}]_i$ in VSMC by stimulating Ca^{2+} influx through the plasma membrane. Sodium nitroprusside (SNP, 10^{-7} M), a NO donor, was added 5 min before the application of KCl when necessary.

Because the emphasis of the present study was on the effect of NO on intracellular Ca^{2+} release, further steps were not taken to investigate the effect of NO in Ca^{2+} entry.

2.2. Effect of NO on Intracellular Ca^{2+} Release.

Phasic contractions in aortic rings (either endothelium-intact or removed) induced by agonists in Ca^{2+}-free solution were used as an indicator of intracellular Ca^{2+} release. Phenylephrine (10^{-6} M) and caffeine (20 mM) were applied to stimulate intracellular Ca^{2+} release through the IP_3 receptor pathway and the ryanodine receptor pathway, respectively. ACh (10^{-5} M) was used to stimulate NO release from the endothelium while sodium nitroprusside (10^{-7} M) was applied as a NO donor.

$[Ca^{2+}]_i$ in primarily cultured VSMC from rat aorta was determined. Phenylephrine (10^{-6} M) was added to induce intracellular Ca^{2+} release in VSMC in Ca^{2+}-free solution. Sodium nitroprusside (10^{-7} M) was applied to see if it could affect the alteration of $[Ca^{2+}]_i$ induced by phenylephrine in Ca^{2+}-free solution.

3. Results

3.1. Effect of NO on Ca^{2+} Entry.

A. Effect of ACh on KCl-induced Contractions.

KCl (30 mM) generated sustained tensions of aortic rings with endothelium in Ca^{2+}-containing solution. The administration of ACh (10^{-5} M) 5 sec before the addition of KCl remarkably attenuated the KCl-induced contraction (Fig.3-1A.). Data from 6 experiments showed that ACh reduced the KCl-induced tension by 57.6% ($P<0.01$,

n=6). The KCl-evoked contraction was not affected by ACh after endothelium was removed (Fig.3-1B).

B. Effect of SNP on Elevation of $[Ca^{2+}]_i$ Induced by KCl.

$[Ca^{2+}]_i$ inVSMC was elevated rapidly and transiently by the administration of KCl (15 mM). In the presence of sodium nitroprusside (10^{-5} M), the elevation of $[Ca^{2+}]_i$ induced by KCl was significantly reduced by 70.5% ($P<0.01$, n=4). After wash, the increment of $[Ca^{2+}]_i$ returned closely to the control level (Fig.3-2.).

3.2. Effect of NO on Intracellular Ca^{2+} Release.

A. Phasic Contractions Induced by Phenylephrine and Caffeine in Ca^{2+}-free Solution.

Both phenylephrine (10^{-6} M) and caffeine (20 mM) evoked rapid and phasic contractions in endothelium-intact or -removed aortic rings in Ca^{2+}-free solution. Muscle tensions reached their maximal levels within 10 seconds and declined thereafter without a sustained contraction. To exclude the possibility that residual Ca^{2+} left in organ chambers might play a role in the contractile responses, KCl (30 mM) which can stimulate Ca^{2+} entry through the plasma membrane was applied after incubation with Ca^{2+}-free solution for 5 minutes, but it could not induce a contractile response of the aortic ring (data not shown). Thus, phasic contractions induced by phenylephrine and caffeine are due to intracellular Ca^{2+} release.

B. Effect of ACh on Phenylephrine- or Caffeine-induced Phasic Contractions in Ca^{2+}-free Solution.

Fig.3-3A. shows a series of records of phenylephrine-induced phasic contractions from one aortic ring with endothelium. In the presence of ACh (10^{-7}, 10^{-6}, and 10^{-5} M), the maximal phasic contraction was reduced from 0.62 g to 0.49 g, 0.44 g, and 0.36 g, respectively. Data from six aortic rings were summarized in Fig.3-3B. ACh at 10^{-7} M slightly inhibited the peak contractile responses by 8.65%, while ACh at 10^{-6} M and 10^{-5} M significantly attenuated the phasic contractions by 25.6% and 30.2%, respectively. However, caffeine-induced phasic contractions were not significantly affected by ACh. Even at 10^{-5} M, ACh could not obviously reduce the caffeine-induced contractile responses in intact aortic rings (Fig.3-4.).

To determine whether the inhibition of phenylephrine-induced phasic contractions by ACh was endothelium-dependent or not, the action of ACh was tested on endothelium-removed aortic rings (Fig.3-5.). Phenylephrine also induced phasic contractions in denuded aortic rings in Ca^{2+}-free solution, which was not influenced by ACh (10^{-5} M), suggesting that the inhibitory effect of ACh is endothelium dependent.

To confirm whether the inhibition by ACh was mediated by NO derived from the endothelium, L-NNA, a potent NOS inhibitor was used. Exposure to L-NNA (10^{-5} M) completely blocked the inhibition by ACh, which could be totally reversed by L-arginine (10^{-4} M) but not by D-arginine (10^{-4} M) (Fig.3-6.). Fig.3-7. shows that methylene blue (10^{-5} M), a soluble guanylate cyclase inhibitor, also completely antagonized the effect of ACh. Taken together, our data suggest that the NO:cyclic GMP pathway is responsible for the inhibition of phenylephrine-induced phasic contractions by ACh in Ca^{2+}-free solution.

C. Effect of Sodium Nitroprusside on Phenylephrine- or Caffeine-induced Phasic Contractions in Denuded Aortic Rings.

Sodium nitroprusside is a NO donor which can spontaneously release NO in a physiological salt solution (Moncada et al., 1991). In the presence of SNP (10^{-7} M), the phenylephrine-evoked phasic contractions in denuded aortic rings in Ca^{2+}-free solution were significantly attenuated by 96.8%, which could be incompletely reversed by methylene blue (5×10^{-5} M) (Fig.3-8A.). Higher concentration of methylene blue did not show bigger reversal effect (data not shown). However, SNP could not significantly inhibit the caffeine-induced phasic contractions in denuded aortic rings (Fig.3-8B.)

D. Effect of SNP on Phenylephrine-induced Elevation of $[Ca^{2+}]_i$ in VSMC.

Phenylephrine (10^{-6} M) evoked a quick and temporary rise in the fluorescence ratio (F340/F380) recorded in cultured VSMC in Ca^{2+}-free 5K solution, indicating a rapid and transient elevation of $[Ca^{2+}]_i$. This response was repeated provided the cells were incubated for 5 min with Ca^{2+}-containing 5K solution prior to the incubation with Ca^{2+}-free solution. The second such response was absent without the preincubation with Ca^{2+}-containing 5K solution (Fig.3-9.). Three dishes showed similar results, suggesting that refilling of the phenylephrine-sensitive Ca^{2+} store was dependent on the extracellular Ca^{2+}, and there is not sufficient residual Ca^{2+} in Ca^{2+}-free 5K solution to increase $[Ca^{2+}]_i$ via Ca^{2+} entry through the plasma membrane. Thus, the transient rise in $[Ca^{2+}]_i$ induced by phenylephrine in Ca^{2+}-free solution is most possibly due to intracellular Ca^{2+} release. Exposure to sodium nitroprusside (10^{-7} M) significantly reduced the phenylephrine-evoked increase in $[Ca^{2+}]_i$ by 66.5%, which could be

partially reversed by methylene blue (10^{-5} M) (Fig.3-10.). These results suggest that NO inhibits intracellular Ca^{2+} release induced by phenylephrine in VSMC.

4. Discussion

The current study showed that ACh attenuated KCl-induced contractions in aortic rings with endothelium, and reduced the elevation of $[Ca^{2+}]_i$ evoked by KCl in VSMC, suggesting that NO inhibits Ca^{2+} entry. These findings are consistent with the previous results (Blatter and Wier, 1994).

It is accepted that the ER is the major intracellular source of Ca^{2+} which makes significant contributions to the regulation of cytoplasmic Ca^{2+} in smooth muscle (Somlyo and Himpens, 1989). Two kinds of receptors regulating Ca^{2+} release have been described on the membrane of the ER: the IP_3 receptor and the ryanodine receptor (Missiaen et al., 1992). Phenylephrine is an α_1-adrenoreceptor agonist which has been shown to induce a rapid transient increase in IP_3 formation in VSM of rat aorta (Langlands and Diamond, 1990). Subsequently, IP_3 stimulates the release of Ca^{2+} from internal stores, leading to contraction of VSM (Somlyo et al., 1985). Caffeine is thought to directly activate the ryanodine receptor and leads to intracellular Ca^{2+} release (Missiaen et al., 1992).

Our data demonstrated that NO inhibited intracellular Ca^{2+} release induced by phenylephrine but not by caffeine. This inhibition was mediated by cyclic GMP. This is

the first time to show that NO selectively interferes with intracellular Ca^{2+} release through a distinct pathway in VSM.

In our preparations, the inhibition of phenylephrine-induced contractile responses by ACh in Ca^{2+}-free solution was shown to be mediated by NO produced in the endothelium. These results suggest that endothelial cNOS can be activated upon stimulation with ACh even in the absence of extracellular Ca^{2+}. It has been well known that endothelial NOS is activated by transient elevation in Ca^{2+} (Förstermann et al., 1994) induced by agonists, via Ca^{2+} entry through the plasma membrane and the release of internal Ca^{2+} stores (Schilling et al., 1992; Jacob, 1990). ACh has been shown to cause a transient release of Ca^{2+} from an intracellular store in freshly isolated endothelial cells (Wang et al., 1995). This result therefore provided an explanation for the observation that endothelial cNOS was activated by ACh in Ca^{2+}-free solution.

Sodium nitroprusside is a commonly used NO donor. At 10^{-7} M, sodium nitroprusside demonstrated a much stronger inhibitory effect on phenylephrine-induced phasic contractions than ACh (10^{-5} M) did, and methylene blue (10^{-5} M) only partially blocked the inhibition by sodium nitroprusside. Two possibilities are proposed to explain this phenomenon. First, since endothelial cNOS is Ca^{2+}-dependent, its activity could be reduced in Ca^{2+}-free solution. Previous studies described that cyclic GMP production was obviously attenuated in rat thoracic aorta in response to ACh after removal of extracellular Ca^{2+} (Moritoki et al., 1994). Second, sodium nitroprusside dissociates spontaneously in solution to NO and other compounds (Moncada et al., 1991). The possibility that sodium nitroprusside also exerted its role through other

pathways besides NO could not be completely excluded. If this was the case, it was not surprising that sodium nitroprusside had greater inhibition than ACh, and its action could not be totally blocked by methylene blue.

The contractile activity of VSM is a complicated process. It is certainly associated with the elevation of $[Ca^{2+}]_i$ and interaction between different proteins. Thus, any alteration in $[Ca^{2+}]_i$ or contractile apparatus may lead to change in contraction. In our preparations, both endogenous and exogenous NO attenuated the phasic contractions of vascular smooth muscle induced by phenylephrine but not by caffeine, suggesting that NO is unlikely to significantly interfere with the contractile apparatus in these preparations. In other words, the inhibitory effect of NO is most probably due to the reduction of $[Ca^{2+}]_i$ which is mainly realized by inhibiting intracellular Ca^{2+} release in the absence of extracellular Ca^{2+}. Cyclic GMP was demonstrated to mediate this inhibition, in accordance with previous results that cyclic GMP inhibited agonist-evoked intracellular Ca^{2+} release presumably by inhibiting the effects of IP_3 on the ER (Komalavilas and Lincoln, 1994) as well as IP_3 generation in smooth muscle cells (Ruth et al., 1993).

$[Ca^{2+}]_i$ in VSMC was measured in order to further confirm that NO (released from SNP) can affect intracellular Ca^{2+} release induced by phenylephrine. Phenylephrine evoked a rapid elevation of $[Ca^{2+}]_i$ in VSMC in Ca^{2+}-free solution. Exposure to sodium nitroprusside significantly reduced this elevation, suggesting that NO inhibits intracellular Ca^{2+} release induced by phenylephrine. However, as in the tension study, the existence of other mechanisms by which sodium nitroprusside performed its

inhibitiory role could not be absolutely ruled out since methylene blue only partially blocked the action of sodium nitroprusside.

Accumulating evidence has demonstrated differences between the effects of NO on intracellular Ca^{2+} release in various tissues. NO was reported to mobilize intracellular Ca^{2+} by selectively activating a Ca^{2+} signal pathway involving ryanodine receptor-like Ca^{2+} channels on the ER while having no effect on the IP_3 pathway in sea urchin eggs (Willmott et al., 1996). Other results also showed that NO and NO donors elicited increases in $[Ca^{2+}]_i$ in interstitial cells in the mammalian gut (Publicover et al., 1993), a macrophage line (Kong et al., 1994), and pancreatic β cells (Willmott et al., 1995). EDRF was found to inhibit intracellular Ca^{2+} release in rabbit aorta (Collins et al., 1986). NO donors were shown to block bile canalicular contraction by inhibiting IP_3-dependent Ca^{2+} mobilization (Dufour et al., 1995). However, the NO donor was also found to have no effect on vasopressin-induced Ca^{2+} release from internal stores in VSMC (Blatter and Wier, 1994). Therefore, the effects of NO on intracellular Ca^{2+} release seem to be dependent on tissue types and agonists used. Our observation that NO selectively inhibits intracellular Ca^{2+} release is a new support for this assumption. In addition, our findings also provide evidence for an alternative mechanism by which NO inhibits agonist-induced contraction in VSM.

In conclusion, NO may regulate contractility of VSM via altering $[Ca^{2+}]_i$. NO not only inhibits Ca^{2+} entry through the plasma membrane but also selectively inhibits intracellular Ca^{2+} release through a distinct pathway in VSM.

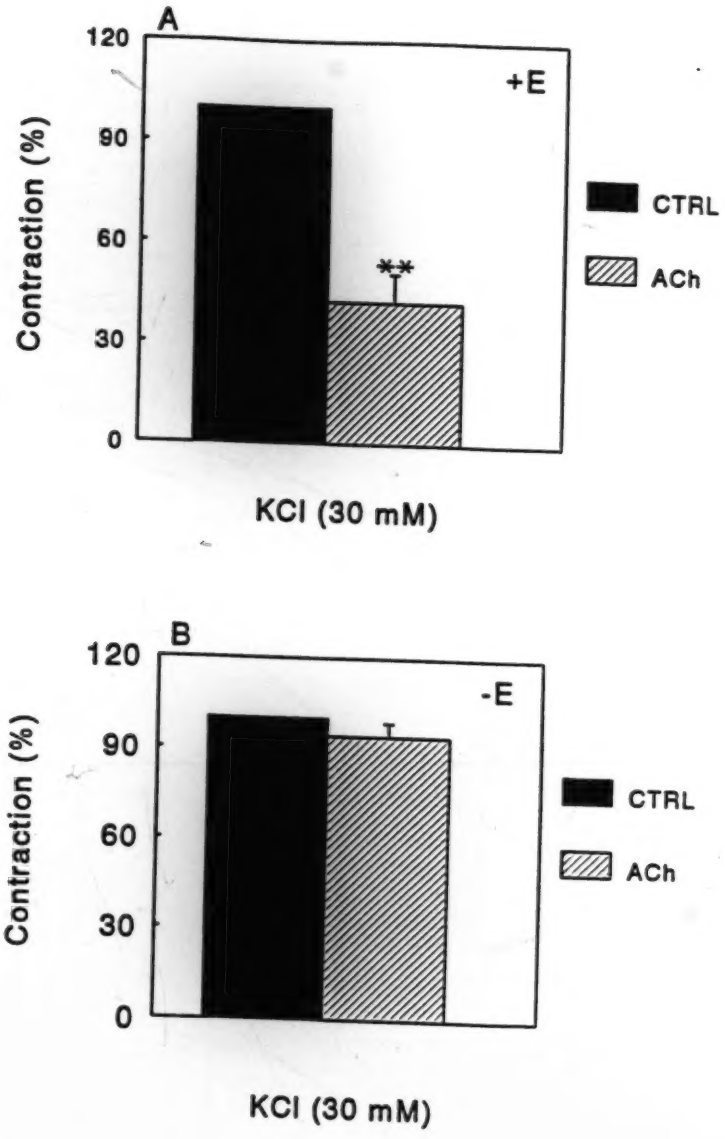

Fig.3-1. Effects of ACh on KCl-induced contractions in aortic rings with (A) or without (B) endothelium in Ca^{2+}-containing solution. ACh (10^{-5} M) was applied 5 sec before the administration of KCl (30 mM). The values of maximal phasic contractions under control conditions were taken as 100% while other values were normalized to their control. Vertical bars, S.E.. **, significant difference aganist control. +E, with endothelium. -E, without endothelium. n=6.

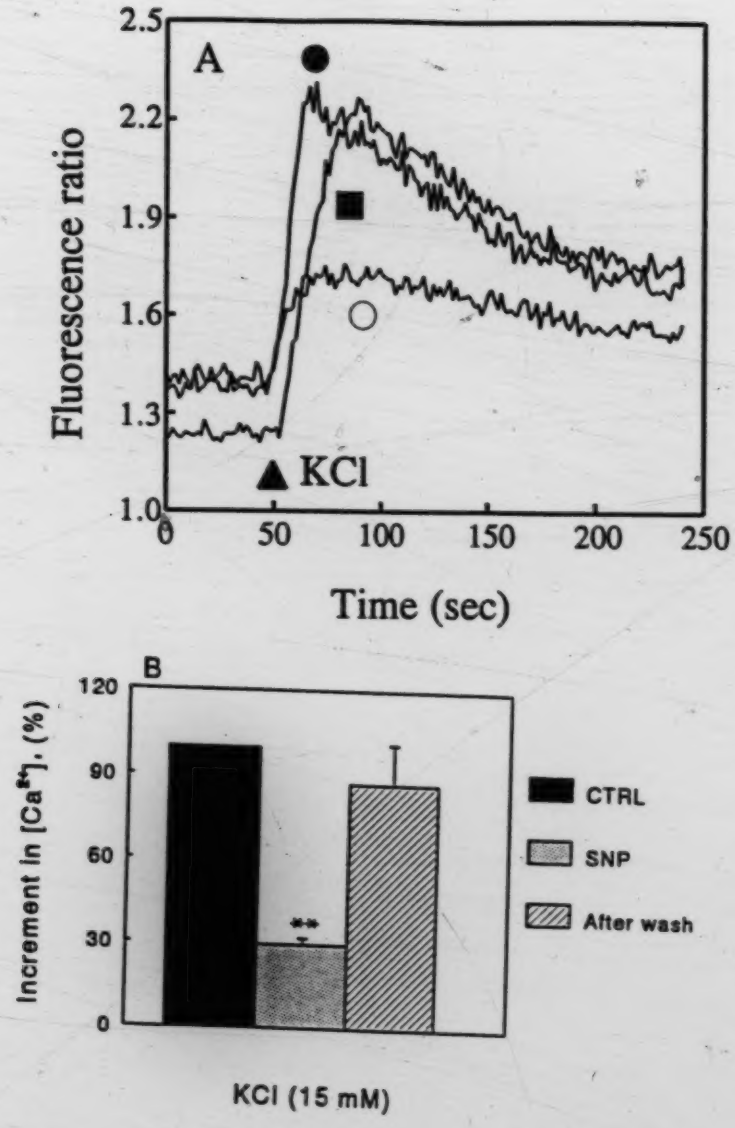

Fig.3-2. Effect of SNP on KCl-evoked elevation of $[Ca^{2+}]_i$ in VSMCs in Ca^{2+}-containing solution. SNP was applied 5 min before the KCl (15 mM) was added. A. Original records from one dish of VSMCs showing the elevation of $[Ca^{2+}]_i$ induced by KCl in control (●), in the presence of SNP (10^{-7} M , ○), and after wash (■). B. Data from four dishes of VSMCs were summarized. Maximal increased in $[Ca^{2+}]_i$ (control) were taken as 100% while other values were normalized to their control. Vertical bars, S.E.. ** , significant difference against control. SNP, sodium nitroprusside. n=4.

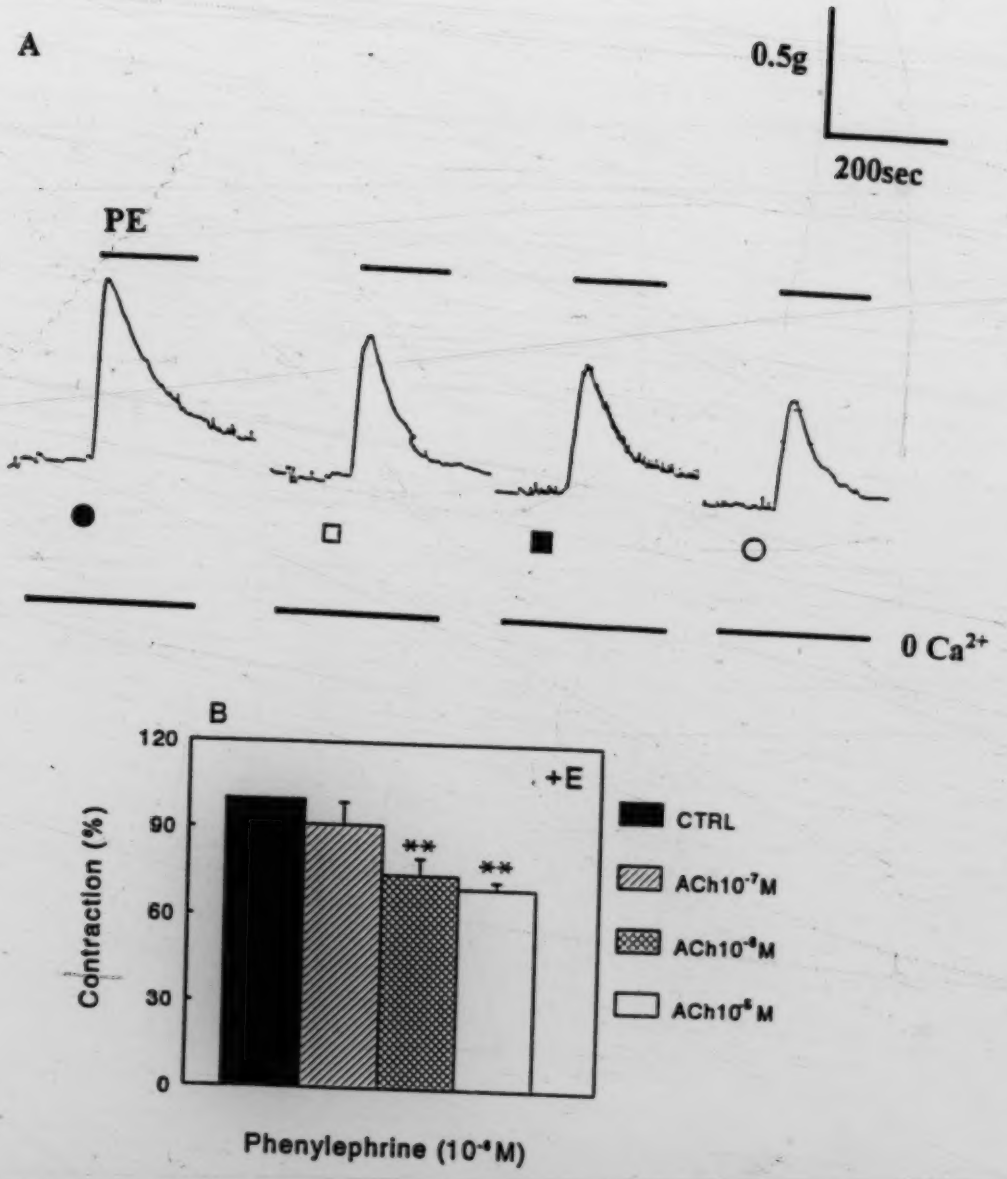

Fig.3-3. Effect of ACh on phenylephrine-evoked phasic contractions in endothelium-intact aortic rings in Ca^{2+}-free solution. A. Original records obtained from one aortic ring showing phasic contractions induced by phenylephrine (10^{-6} M) in the absence of ACh (●) and in the presence of ACh (□, 10^{-7} M; ■, 10^{-6} M, and ○, 10^{-5} M, respectively). ACh was added just 5 sec before the administration of phenylephrine. 0 Ca^{2+}, in the absence of extracellular Ca^{2+}. PE, phenylephrine. B. Six experiments were summarized in histogram. The values of maximal phasic contractions under control conditions were taken as 100% whereas other tension values in the presence of ACh with different concentrations were normalized to their control. Vertical bars, S.E.. **, significant difference against control. +E, with intact endothelium. CTRL, control. n=6.

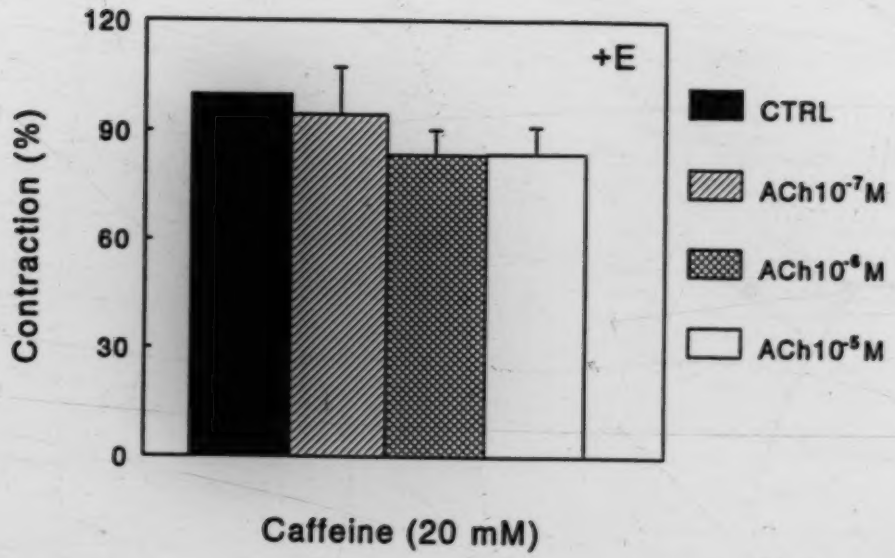

Fig.3-4. Effect of ACh on caffeine-induced phasic contractions in endothelium-intact aortic rings in Ca^{2+}-free solution. ACh was added 5 sec before the administration of caffeine (20 mM). Maximal phasic tension values under control conditions were taken as 100% while other values in the presence of different concentrations of ACh were normalized to their control. Vertical bars, S.E.. +E, with intact endothelium. CTRL, control. n=6.

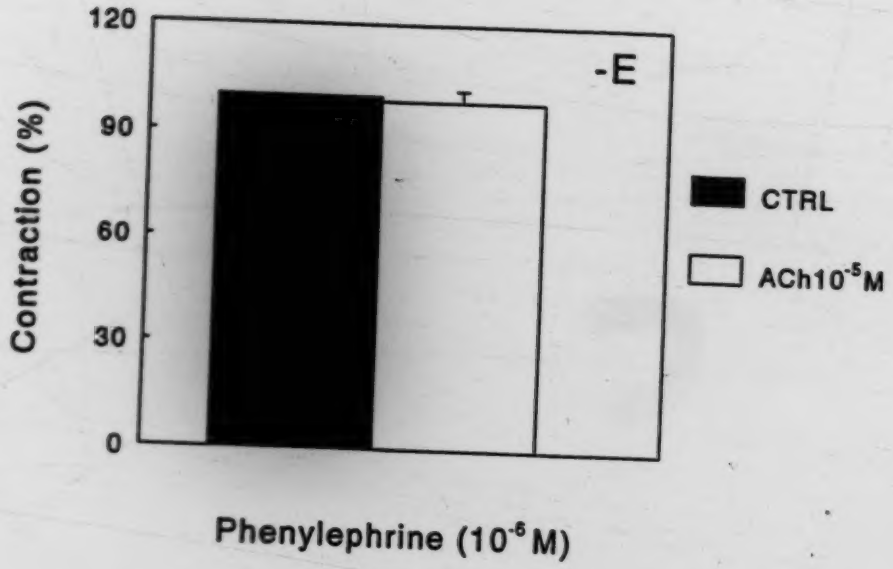

Fig.3-5. Effect of ACh on phenylephrine-induced phasic contractions in endothelium- denuded aortic rings in Ca^{2+}-free solution. ACh (10^{-5} M) was added 5 sec before the application of phenylephrine (10^{-6} M). The values of maximal phasic contractions under control conditions were taken as 100% while other values in the presence of ACh were normalized to their control. Vertical bars, S.E.. -E, without endothelium. CTRL, control. n=6.

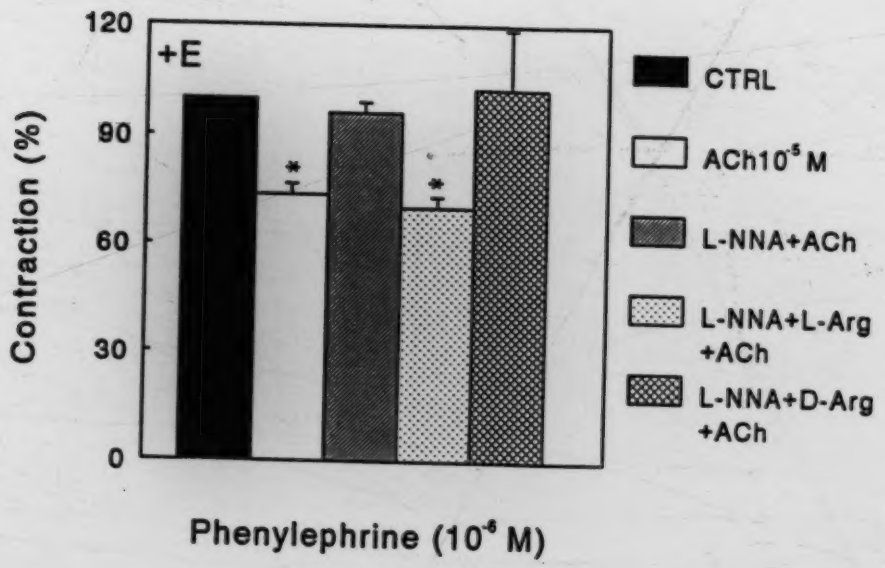

Fig.3-6. Effect of ACh on phenylephrine-evoked phasic contractions in endothelium-intact aortic rings in Ca^{2+}-free solution in the presence of L-NNA, L-NNA+L-arginine or L-NNA+D-arginine, respectively. ACh (10^{-5} M) was added 5 sec before the administration of phenylephrine (10^{-6} M). Aortic rings were exposed to L-NNA (10^{-3} M), L-NNA+L-arginine (10^{-4} M) or L-NNA+D-arginine (10^{-4} M) when they were incubated in Ca^{2+}-free solution. The values of maximal phasic contractions under control conditions were taken as 100% while other values were normalized to their control. Vertical bars, S.E.. * $P<0.05$ against control. +E, with intact endothelium. L-arg, L-arginine. D-arg, D-arginine. n=6.

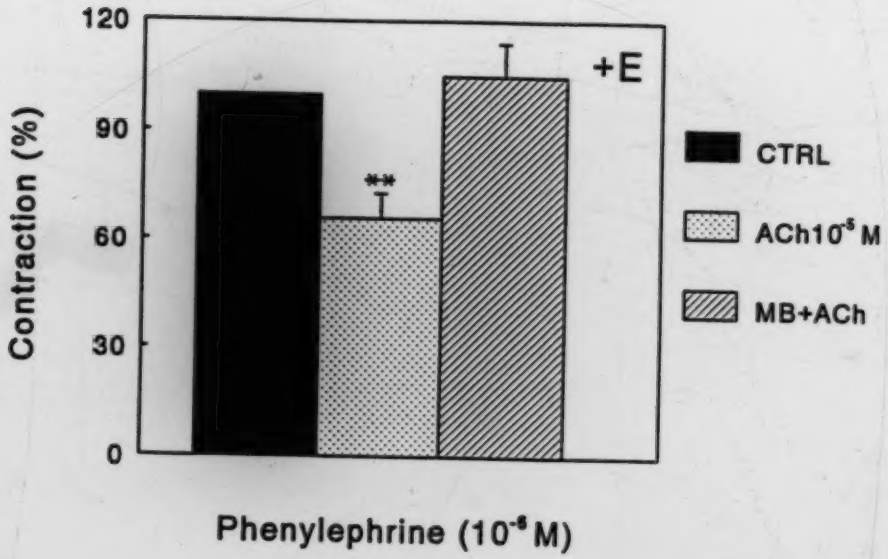

Fig.3-7. Effect of ACh on phenylephrine-induced phasic contractions in endothelium- intact aortic rings in the presence of methylene blue in Ca^{2+}-free solution. ACh (10^{-5} M) was added 5 sec before the administration of phenylephrine (10^{-6} M). Exposure of aortic rings to methylene blue (10^{-5} M) began at the same time as they were incubated in Ca^{2+}-free solution. The values of maximal phasic contractions under control conditions were taken as 100% while other values were normalized to their control. Vertical bars, S.E.. **, significant difference against control. +E, with intact endothelium. MB, methylene blue. n=6.

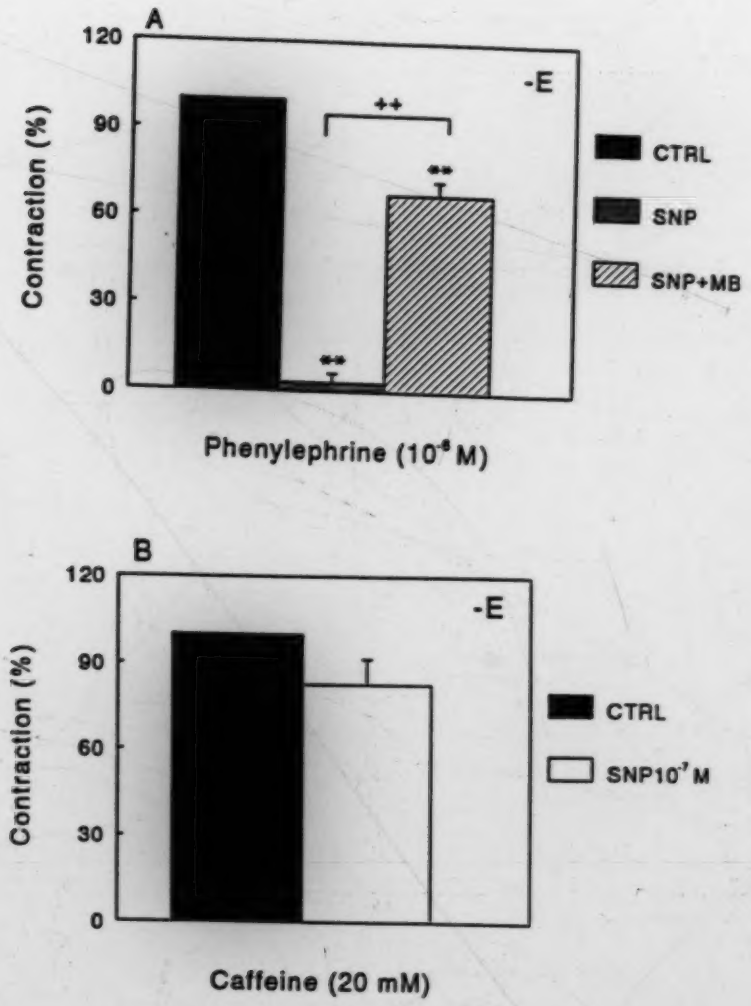

Fig.3-8. Effects of SNP on phenylephrine- or caffeine-induced phasic contractions in endothelium-denuded aortic rings in Ca^{2+}-free solution. SNP (10^{-7} M), or SNP with methylene blue (10^{-5} M) were added 5 min before the administration of phenylephrine (10^{-6} M) or caffeine (20 mM). The values of maximal phasic contractions under control conditions were taken as 100% while other values were normalized to their control. A: Effect of SNP on phenylephrine-evoked phasic contractions; B: Effect of SNP on caffeine-induced phasic contractions. Vertical bars, S.E.. **, significant difference against control. ++, significant difference between in the presence of SNP and in the simultaneous presence of SNP and methylene blue. -E, without endothelium. MB, methylene blue. SNP, sodium nitroprusside. n=6.

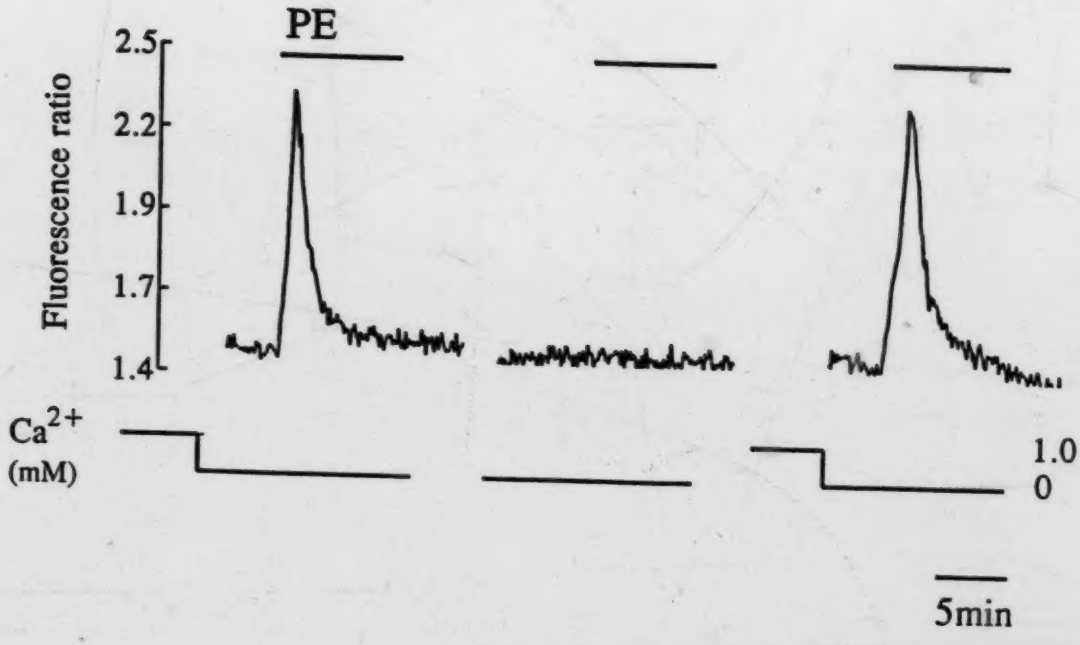

Fig.3-9. Dependence of refilling of the phenylephrine-sensitive Ca^{2+} store on the extracellular Ca^{2+} source. Original consecutive records of fluorescence ratio (F340/F380) obtained from one dish of VSMCs. Phenylephrine (10^{-6} M) was added 5 min after incubation with Ca^{2+}-free 5K solution following a 5 min pre-incubation with Ca^{2+}-containing or Ca^{2+}-free 5K solution. PE, phenylephrine. Ca^{2+}, extracellular Ca^{2+} concentration.

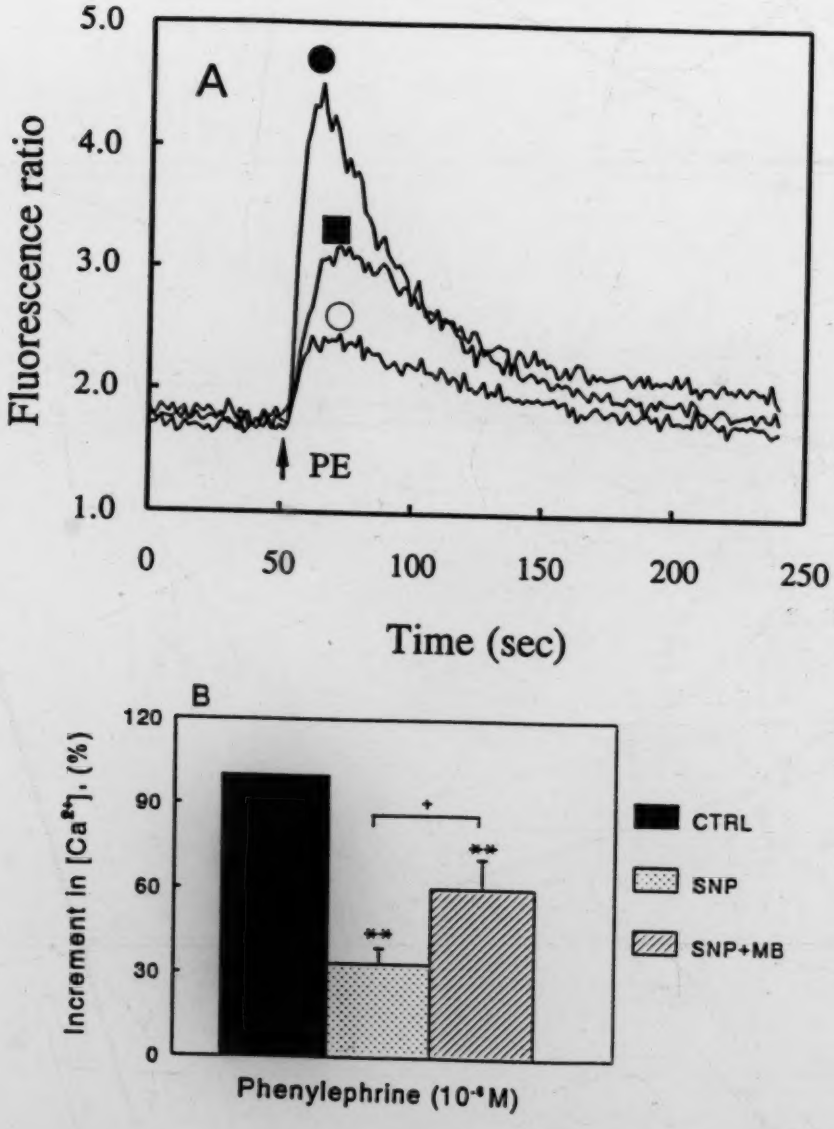

Fig.3-10. Effect of SNP on phenylephrine-induced elevation of $[Ca^{2+}]_i$ in VSMCs in Ca^{2+}-free 5K solution. SNP (10^{-7} M) or SNP with methylene blue (10^{-5} M)was added at the beginning of incubation with Ca^{2+}-free 5K solution and 5 min before the administration of phenylephrine (10^{-6} M). A. Original records from one dish of VSMCs showing the elevation of $[Ca^{2+}]_i$ induced by phenylephrine in control (●), in the presence of SNP (○), and in the co-presence of SNP and methylene blue (■). PE, phenylephrine. B. Data from five dishes of VSMCs were summarized. Maximal increase in $[Ca^{2+}]_i$ under control conditions were taken as 100% whereas other values were normalized to their control. Vertical bars, S.E.. ** , significant difference against control, $^+$, $P<0.05$ between in the presence of SNP and in the simultaneous presence of SNP and methylene blue. CTRL, control. MB, methylene blue. SNP, sodium nitroprusside. n=5.

CHAPTER IV. NOS ACTIVITY IN VSM

1. Introduction

NO plays an important role in the regulation of vascular tone by stimulating soluble guanylate cyclase, which in turn leads to an accumulation of guanosine 3', 5'- cyclic monophosphate (cGMP) in VSMC (Moncada et al., 1991). NO is formed from the amino acid L-arginine (Palmer et al., 1988b) by nitric oxide synthase (NOS). So far three isoforms of NOS have been identified (Förstermann et al., 1994). Isoform I and isoform III are usually expressed constitutively in brain and endothelial cells, respectively, and are also termed constitutive nitric oxide synthase (cNOS). Isoform II of NOS is not constitutively expressed, but it can be induced in macrophages, smooth muscle cells and many other cells with bacterial lipopolysaccharide and/or cytokines. This enzyme is also termed inducible nitric oxide synthase (iNOS).

It has been well established that endotoxin and cytokines induce iNOS messenger RNA expression in a time-dependent manner in cultured VSMCs from aortic (Weigert et al., 1995; Kanno et al., 1993; Durante et al., 1996) or pulmonary (Wong et al., 1995; Nakayama et al., 1994) artery and in aortic strips (Sirsjo et al., 1994) followed by a prolonged production of NO. In all these studies, iNOS messenger RNA and significant amount of NO was not detected in the same preparations without stimulation with endotoxin and/or cytokines. In other investigations, NO formation was detectable in vascular smooth muscle (or cells) without stimulation with endotoxin and/or cytokines

(under basal conditions). Bernhardt et al. (1991) reported that continuous synthesis of NO in human VSMC from mesenteric artery was detectable in an endotoxin-free environment. Zehetgruber et al. (1993) showed that NO was produced by VSM from bovine pulmonary artery without exposure to endotoxin and/or cytokines as detected by chemiluminence, but the amount of NO released was insufficient to relax endothelium-deprived vascular preparations. These results indicate that there is little NOS activity in vascular smooth muscle and it is too low to influence tension generation under basal conditions.

Previous results showed that phosphorylation of NOS in neurons (Maiese et al., 1993), endothelial cells (Hirata et al., 1995) and gastric smooth muscle cells (Murphy et al., 1994) by protein kinase C (PKC) resulted in the reduction of NOS activity. Consensus sequences for phosphorylation by cAMP-dependent protein kinase (PKA) were evident in brain NOS, endothelial NOS and hepatic-inducible NOS (Bredt and Snyder, 1994). Based on these facts, we hypothesized that NOS activity in vascular smooth muscle under basal conditions was probably inhibited by protein kinases. If this was the case, removal of this inhibition would lead to an increase in NOS activity which could therefore affect tension development of vascular smooth muscle. In the current study, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), a non-specific inhibitor of protein kinases, was employed to examine whether its inhibitory effect on the phenylephrine-induced contractions in endothelium-removed aortic rings was mediated by NO synthesized in VSM.

In addition, inducible NOS activity in VSM was also tested after prolonged exposure of denuded aortic rings to endotoxin lipopolysaccharide (LPS).

2. Experimental Design

2.1. To Study the Effect of Pretreatment of Denuded Aortic Rings with LPS on Vascular Contractility.

It has been known that NOS can be stimulated to express in VSM after prolonged exposure of VSM to endotoxin lipopolysaccharide (LPS). Aortic rings without endothelium were preincubated for 6 hrs in MEM containing LPS (3, 30, or 300 ng/ml). Other denuded aortic rings preincubated in MEM without LPS were taken as control group. These arterial tissues were then mounted in organ chambers and washed with Krebs solution at least 3 times to remove residual LPS. After the arterial tissues were stable, serial concentration-contractions induced by phenylephrine (10^{-9} to 10^{-5} M) were obtained. The interval between the adjacent concentration-contractions was 30 min.

By comparing the phenylephrine-induced concentration-dependent contractile responses between denuded aortic rings pretreated and non-pretreated with LPS, it could be determined whether preincubation with LPS had an effect on vascular contractility. To further confirm whether the effect was mediated by NOS-produced NO, NOS inhibitor L-NNA (10^{-5} M), alone or with L-arginine (10^{-4} M) was used.

2.2. To Study whether NOS in VSM under Basal Conditions Was Inhibited by Protein Kinases.

Consecutive concentration-contractions in denuded aortic rings induced by phenylephrine (10^{-9} to 10^{-5} M) were recorded with an interval of 30 min. H-7 (10^{-6} to 10^{-5} M) was added during the interval to show its effect on the phenylephrine-induced concentration-contractions. To determine whether the action of H-7 was mediated by NO:cyclic GMP pathway, L-NNA (10^{-5} M), or aminoguanidine (10^{-4} M) alone or with L-arginine (10^{-4} M) or D-arginine (10^{-4} M) were applied during the interval to see if they could block the effect of H-7. Methylene blue (5×10^{-5} M) was added during the last 10 min in the interval.

3. Results

3.1. Effect of Pretreatment with LPS on Vascular Contractility

Pretreatment with LPS (3, 30, and 300 ng/ml) significantly reduced the phenylephrine-induced concentration-contractions in denuded aortic rings in a dose-dependent manner, and at 10^{-5} M phenylephrine, the contractions were attenuated from 1.36±0.07 g (control) to 0.99±0.08 g, 0.82±0.12 g and 0.66±0.13 g, respectively (Fig.4-1.). L-NNA (10^{-5} M), a NOS inhibitor, significantly blocked the hyporeactivity to phenylephrine after exposure to LPS, which was reversed by L-arginine (10^{-4} M) (Fig.4-2.). However, L-NNA or L-NNA plus L-arginine had no obvious effect on the concentration-response curves in control group (Fig.4-3.). Incubation of denuded

aortic rings with LPS (300 ng/ml) for 1 h did not affect the phenylephrine-evoked concentration-response curves (data not shown.). The data suggest that pretreatment with LPS causes the impairment of vascular contractility which is mediated by NO formation. Since the impaired contractility was shown in organ chambers free of LPS, suggesting NOS proteins have probably expressed in VSM after prolonged exposure to LPS.

3.2. Inhibitory Effect of H-7 on Phenylephrine-induced Concentration-contraction.

Phenylephrine, an α_1-adrenoreceptor agonist, generated concentration-dependent contractions in denuded aortic rings. The concentrations of phenylephrine ranged from 10^{-9} to 10^{-5} M. The EC_{50} value for phenylephrine-induced concentration-response curves was $1.01\pm0.26\times10^{-7}$ M (n=30) and the tension value at 10^{-5} M phenylephrine was 1.16±0.03 g (n=30). In the presence of H-7 (10^{-6}, 5×10^{-6} and 10^{-5} M), the concentration-contractions induced by phenylephrine were significantly reduced a dose-dependent manner (Fig.4-4.). At 10^{-5} M, H-7 significantly shifted the EC_{50} value rightward to $3.24\pm0.44\times10^{-7}$ M (n=30).

Fig.4-5A. shows that the inhibitory effect of H-7 (10^{-5} M) on phenylephrine-induced contractions in denuded aortic rings occurred rapidly after 5 min incubation with H-7. Longer incubation (15 and 30 min) did not show significant bigger inhibition. Fig.4-5B. demonstrates that after incubation with H-7 for 30 min, it was washed out of the medium. This washout totally abolished the inhibitory effect of H-7.

3.3. NO Involvement in the Inhibitory Effect of H-7 on Phenylephrine-induced Concentration-contraction.

In the simultaneous presence of H-7 (10^{-5} M) and L-NNA (10^{-5} M), the inhibitory effect of H-7 on the contractions at 10^{-5} M phenylephrine was blocked by 88% (Fig.4-6.). L-arginine (10^{-4} M), a NOS substrate, totally antagonized the blockade by L-NNA, but D-arginine (10^{-4} M) had no such an effect. The EC_{50} values and tension values at 10^{-5} M phenylephrine were summarized in Table 4-1.

Aminoguanidine (10^{-4} M), a selective inhibitor of inducible NOS, also partially blocked the inhibitory effect of H-7. The response to phenylephrine at 10^{-5} M was significantly increased from 0.54±0.04 g in the presence of H-7 to 0.84±0.04 g in the simultaneous presence of H-7 and aminoguanidine. Compared with the response to phenylephrine at 10^{-5} M (1.12±0.08 g) in the control group, this dose of aminoguanidine abolished the inhibitory effect of H-7 by 52%. Higher doses of aminoguanidine did not show an increased blockade (data not shown). L-arginine (10^{-4} M) but not D-arginine (10^{-4} M) completely reversed the blockade by aminoguanidine (Fig.4-7.).

Methylene blue (5×10^{-5} M), given during the last 10 minutes of incubation period of H-7 (10^{-5} M), greatly reversed the suppression by H-7. The tension developed by phenylephrine at 10^{-5} M was elevated from 0.51±0.07 g in the presence of H-7 to 0.93±0.07 g in the concomitant presence of H-7 and methylene blue ($P<0.01$, $n=6$) (Fig.4-8.).

In the presence of L-NNA (10^{-5} M) or aminoguanidine (10^{-4} M) alone, the phenylephrine-induced concentration-response curves of denuded aortic rings were not obviously altered. Both the EC_{50} value ($2.73\pm0.99\times10^{-8}$ M or $3.2\pm0.82\times10^{-8}$ M, n=6) and the response to phenylephrine at 10^{-5} M (1.10±0.09 g or 1.10±0.10 g, n=6) were not significantly different from those in the control group (EC_{50}: $4.42\pm0.30\times10^{-8}$ M; tension values at 10^{-5} M phenylephrine: 1.06±0.10 g, n=6) (Fig.4-9.).

4. Discussion

Phenylephrine is a well known activator of α_1-adrenergic receptors in vascular smooth muscle. In the present study, it stimulated VSM of aorta to contract in a dose-dependent manner. Our data showed that the minimal concentration of phenylephrine to elicit contractions was around 10^{-8} M. At 5×10^{-6} M to 10^{-5} M phenylephrine, the highest responses were reached.

Our results demonstrated that contractility of VSM was impaired after exposure to LPS for 6 hrs. This impairment could be improved by the NOS inhibitor, and reversed again by L-arginine, suggesting that L-arginine/NO pathway may involve the hyporeactivity induced by pretreatment with LPS. These data are consistent with previous results that denuded aortic rings pretreated with LPS in vitro (Rees et al., 1990b) showed hyporesponse to agonists which was mediated by NO. Vascular contractility was not affected by 1 h exposure to LPS even in a dose of 300 ng/ml, indicating the induction of NOS by LPS is time-dependent. In addition, the impaired

vascular contractility was not LPS-dependent once it was induced. These data suggest that prolonged exposure of denuded aortic rings to LPS leads to the expression of inducible NOS which in turn damage vascular contractility by synthesizing NO in VSM.

It was also shown that the phenylephrine-elicited responses were greatly inhibited by H-7 (10^{-5} M). This was consistent with previous results (Shimamoto et al., 1993). There was a 51% reduction in tension values at 10^{-5} M phenylephrine in denuded aortic rings in the presence of H-7 if all data collecting from 30 aortic rings were calculated together (n=30).

L-NNA is an inhibitor of various isoforms of NOS (Joly et al., 1994). It considerably blocked the inhibitory effect of H-7 on the phenylephrine-induced contractile responses. The blockade by L-NNA was completely reversed by L-arginine but not by D-arginine. These results indicate that the inhibitory effect of H-7 is mediated by NO in vascular smooth muscle. To clarify what isoform of NOS is responsible for the formation of NO in this situation, aminoguanidine, a selective inhibitor of iNOS (Griffiths et al., 1993; Joly et al., 1994) was used to see whether it could attenuate the inhibition by H-7. The dose of aminoguanidine used also partially and significantly reduced the inhibition of contractile responses by H-7, and L-arginine but not D-arginine totally reversed its action. Aminoguanidine abolished the inhibition of maximal tensions by H-7 by 52% while L-NNA did by 88%. Thus, the NO involved in the inhibitory effect of H-7 is produced at least in part by inducible NOS in VSM. Methylene blue, a guanylate cyclase inhibitor, also significantly blocked the effect of H-7, suggesting cyclic GMP may be involved. Taken together, our data show that the

hyporesponsiveness of denuded aortic rings to phenylephrine in the presence of H-7 is mainly mediated by NO-cGMP pathway.

To confirm the conclusions, two possibilities must be ruled out. First, NO from vascular endothelium which reflects a basal activity of constitutive NOS should be excluded. In our experiments, only those denuded aortic rings which did not show any relaxant response to acetylcholine (10^{-5} M) were selected. Furthermore, in the presence of L-NNA or aminoguanidine alone, phenylephrine-induced concentration-response curves were not significantly altered in denuded aortic rings. However, L-NNA did shift the curves to the left in endothelium-intact aortic rings (data not shown). Hence, endothelium-derived NO can be ruled out as having an effect on tension generation in our vascular preparations. Second, direct increment of vascular contractions by L-NNA or aminoguanidine should also be excluded. According to our observations, L-NNA (10^{-3} M) or aminoguanidine (10^{-4} M) did not potentiate basal tensions of denuded aortic rings. Also, these substances did not significantly shift the phenylephrine-induced concentration-response curves. Similar results for L-NNA (Junquero et al., 1993) and for aminoguanidine (Griffiths et al., 1993) have been shown by other investigators.

Rees et al. (1990) showed that contamination of vascular preparations with endotoxin resulted in a time-dependent induction of NOS in rings of rat thoracic aorta with or without endothelium after eight hours. Although endotoxin was not measured, a hyporesponsiveness to phenylephrine over the course of our experiments was not observed. Therefore, the possibility that increased NOS activity observed in the

presence of H-7 was due to the stimulation by residual LPS was minimal in our vascular preparations.

It has been well established that iNOS in VSM is stimulated by cytokines and endotoxin through gene expression. The characteristics of this stimulation are time-dependent and stimulator-independent once iNOS proteins are expressed. These two features were not met with by H-7 in our study. First, incubation of denuded aortic rings with H-7 for different times produced no different effect on the inhibition of phenylephrine-induced responses. Second, washout of H-7 completely abolished its inhibitory effect. Therefore, it is unlikely that H-7 increases the amount of iNOS proteins through altered gene expression. The augmented activity of iNOS in the presence of H-7 may be due to the regulation of already existing NOS in VSM.

H-7 inhibits PKC by acting at its catalytic domain (Hidaka et al., 1984). The catalytic domain of PKC shows striking homology with the ATP-binding sites of the tyrosine- specific kinases and other serine- and threonine-specific kinases. Hence, H-7 inhibits not only PKC but also a variety of protein kinases such as cGMP-dependent protein kinase (PKG) and PKA (Rüegg and Burgess, 1989). Our data demonstrated that the hyporesponsiveness in the presence of H-7 was mediated by NO, indicating that NOS activity in VSM becomes apparent in the presence of H-7, and its activity is likely to be inhibited by protein kinases in VSM under normal conditions. This inference in supported by the evidence that NOS activity can also be regulated by protein kinases. Consensus sequences for phosphorylation by PKA are evident in brain NOS, endothelial NOS and hepatic-inducible NOS which may represent the prototype

for non-macrophage-inducible NOS (Bredt and Snyder, 1994). Phosphorylation by PKC of brain NOS (Bredt et al., 1992) and endothelial NOS (Lewis and Henderson, 1987) leads to a decrease in NOS activity, whereas phosphorylation by PKA of brain NOS has no effect on NOS activity (Bredt et al., 1992; Brüne and Lapetina, 1991).

In the present study, it is unclear which class of protein kinase is responsible for this inhibition. In addition, the possibility that destruction of NO is slowed down in the presence of H-7 as opposed to direct enhancement of the activity of NOS by H-7 cannot be discriminated. Since H-7 is a non-selective inhibitor of protein kinases, it is not surprising that the NO-cyclic GMP pathway cannot explain the total inhibitory effect of H-7. Other pathways may also be involved. Weak inhibition of myosin light chain kinase by H-7 (Hidaka et al., 1984) is one of possible considerations.

This is the first time to show that NOS activity in VSM is probably inhibited by protein kinases under basal conditions. This inhibition is removed in the presence of H-7, and NOS activity which is minimally in normal situations becomes obvious in a short time and contributes to the regulation of force development. Our findings also provide a new explanation for the pharmacological effect of H-7 on agonist-induced vascular contraction.

It should be emphasized that the hyporesponsiveness of aortic rings observed after exposure to LPS and in the presence of H-7 was caused through different pathways. Although the impairment of phenylephrine-induced contractions was mediated by NO in both cases, LPS was shown to induce the expression of NOS in VSM while H-7 just removed the inhibition of existing NOS activity by protein kinases. This was consistent

with the two differences observed between LPS and H-7. The impairment of vascular contractility after preincubation with LPS was time-dependent and stimulator-independent once it was establised, whereas the hyporeactivity in the presence of H-7 appeared in a short time and was abolished after washout of H-7.

We conclude: (1) the hyporesponsiveness to phenylephrine in the presence of H-7 is mainly mediated by the NO-cGMP pathway; (2) the involved NO is produced by existing NOS in VSM; (3) the NOS activity in VSM is masked probably due to its inhibition by protein kinases under basal conditions.

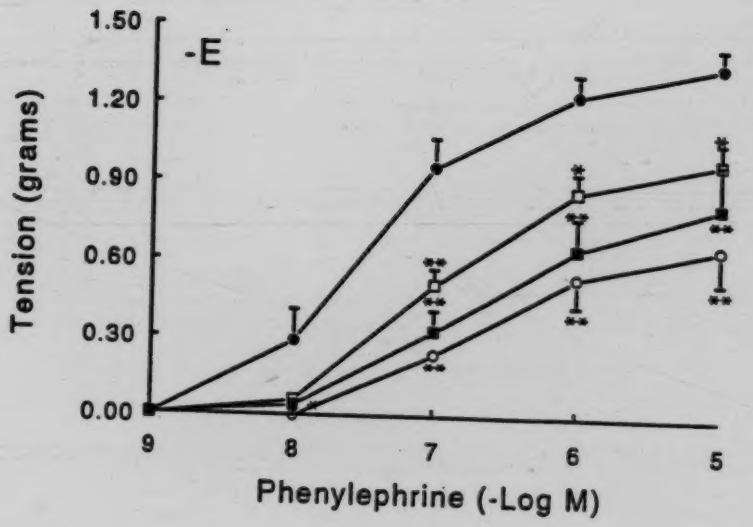

Fig.4-1. Effect of preincubation with LPS on phenylephrine-induced concentration-contraction in endothelium-denuded aortic rings. The rings were preincubated in MEM in the absence (●), or in the presence of LPS (3 ng/ml, □; 30 ng/ml, ■; and 300 ng/ml, ○) for 6 hrs. Vertical bars, S.E.. * or **, significant difference against control. -E, without endothelium. n=6.

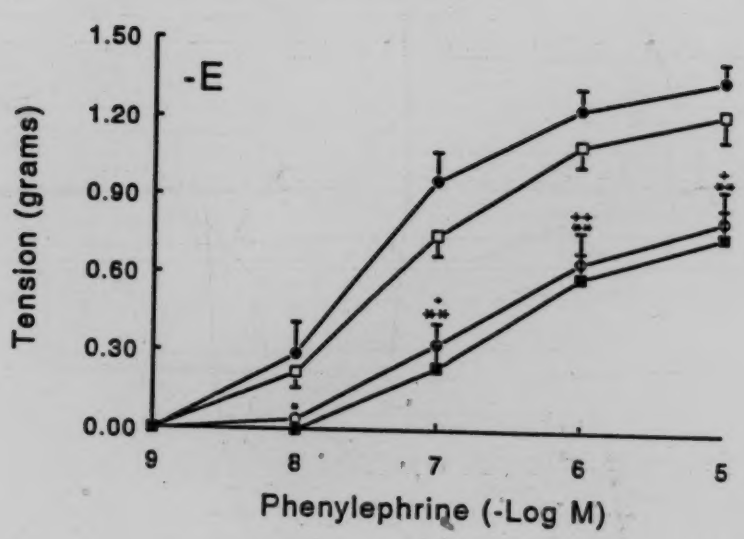

Fig.4-2. Effect of preincubation with LPS on phenylephrine-induced concentration-contraction in endothelium-denuded aortic rings in the presence of L-NNA or L-NNA plus L-arginine. The rings were incubated in MEM in the absence (●), or presence of LPS (300 ng/ml) for 6 hrs. LPS-treated aortic rings were contracted by cumulative concentrations of phenylephrine in the absence (○) and presence of L-NNA (10^{-5} M, □) or in the presence of L-NNA plus L-arginine (10^{-4} M, ■). Vertical bars, S.E.. * or **, significant difference against control. $^{+}$ or $^{++}$, significant difference compared between in the absence and in the presence of L-NNA after exposure to LPS. -E, without endothelium. n=6.

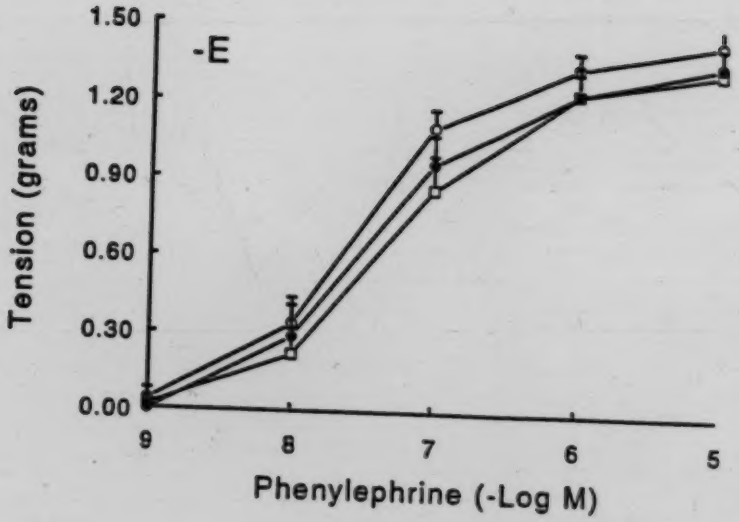

Fig.4-3. Effect of L-NNA and L-NNA plus L-arginine on phenylephrine-induced concentration-contraction in denuded aortic rings. The rings were incubated in MEM for 6 hrs. The aortic rings were contracted by cumulative concentrations of phenylephrine in the absence (●) and presence of L-NNA (10^{-5} M, ○) or in the presence of L-NNA plus L-arginine(10^{-4} M, □). Vertical bars, S.E.. -E, without endothelium. n=6.

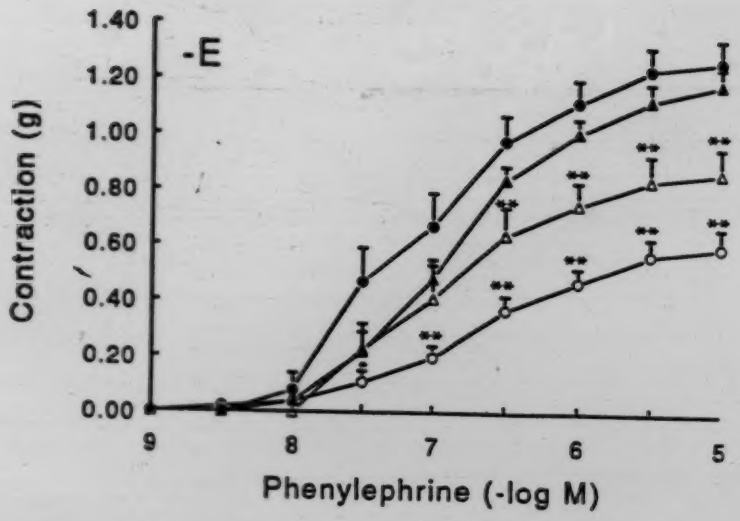

Fig.4-4. Effect of H-7 on phenylephrine-induced concentration-contraction in denuded aortic rings. Concentration-response curves for phenylephrine in control (●, n=6) and in the presence of H-7 (▲, 10^{-6} M; △, 5×10^{-6} M; ○, 10^{-5} M; n=6). Vertical bars, S.E.. * or **, significant difference against control. -E, without endothelium.

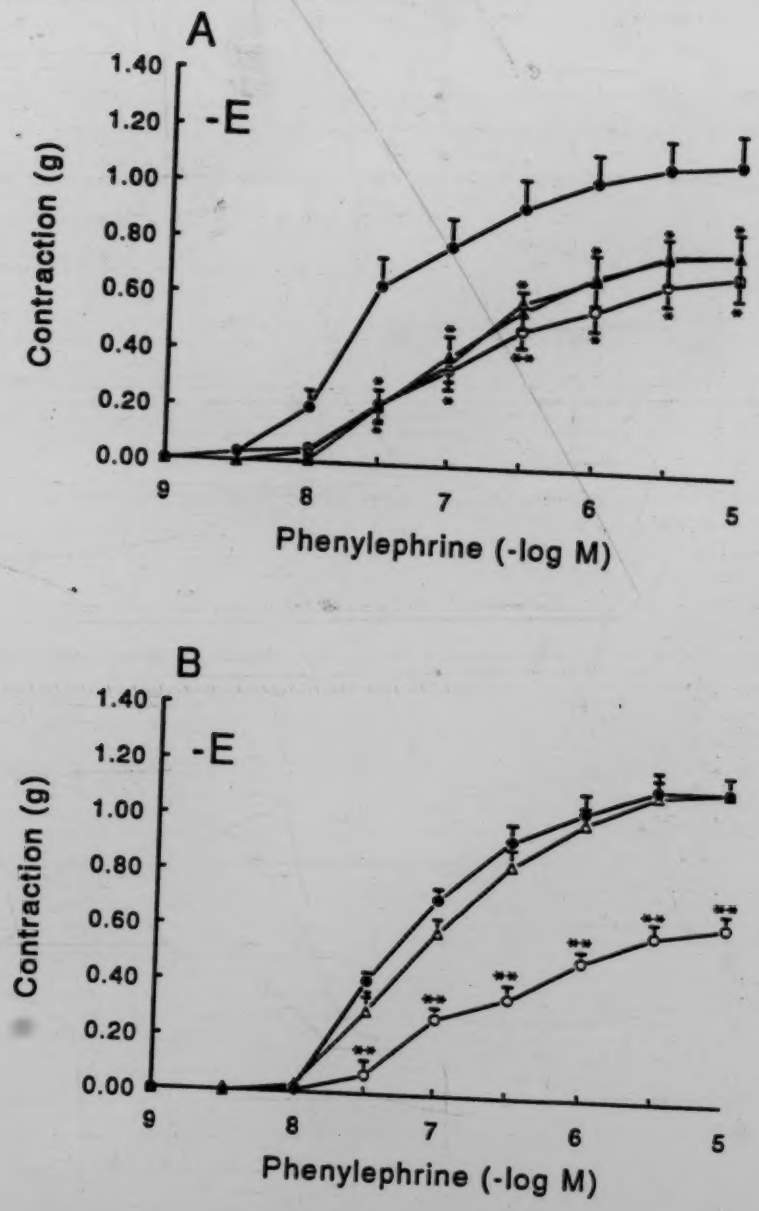

Fig.4-5. Effect of H-7 on phenylephrine-induced concentration-contraction in denuded aortic rings. A: Concentration-response curves for phenylephrine in the absence (●, n=6), and in the presence of H-7 (10^{-5} M) with different incubation periods (▲, 5 min; Δ, 15min; O, 30 min; n=6). B: Concentration-response curves for phenylephrine in control (●, n=6), in the presence of (O, n=6) or after washout (Δ, n=6) of H-7 (10^{-5} M). Vertical bars, S.E.. * or ** , significant difference against control. -E, without endothelium.

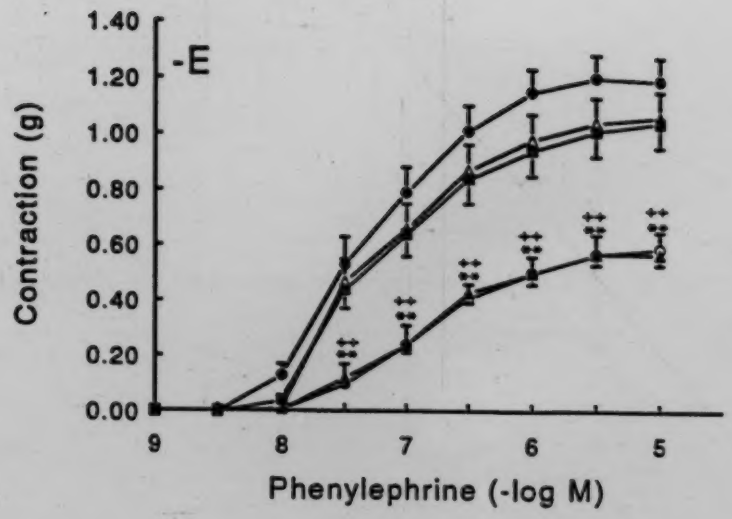

Fig.4-6. Effect of H-7 on phenylephrine-induced concentration-contraction in denuded aortic rings in the presence of L-NNA. Phenylephrine-induced concentration-response curves of denuded aortic rings in control (●, n=6), in the presence of H-7 (10^{-5} M, ○, n=6), in the simultaneous presence of H-7 and L-NNA (10^{-5} M, △, n=6) and in the simultaneous presence of H-7 and L-NNA with L-arginine (10^{-4} M, ▲, n=6) or with D-arginine (10^{-4} M, ■, n=6). Vertical bars, S.E.. * or **, significant difference against control; ++, significant difference between presence of H-7 and simultaneous presence of H-7 and L-NNA. -E, without endothelium.

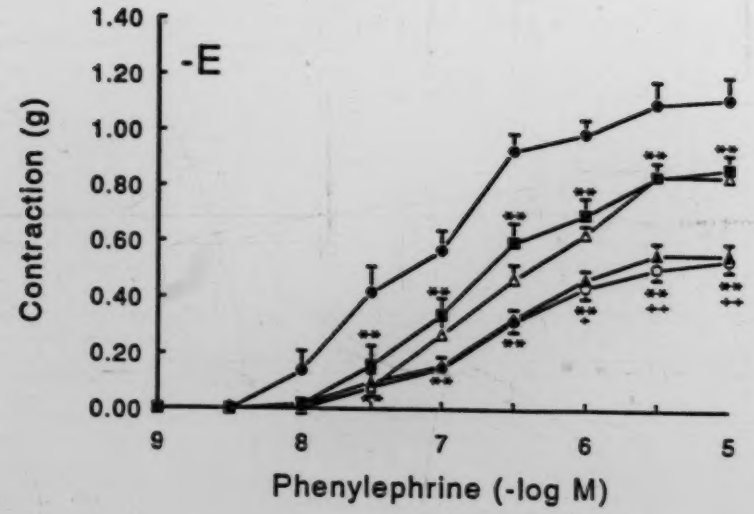

Fig.4-7. Effect of H-7 on phenylephrine-induced concentration-contraction in denuded aortic rings in the presence of aminoguanidine. Phenylephrine-induced concentration-response curves of denuded aortic rings in control (●, n=6), in the presence of H-7 (10^{-5} M, ○, n=6), in the simultaneous presence of H-7 and aminoguanidine(10^{-4} M, Δ, n=6) and in the simultaneous presence of H-7 and aminoguanidine with L-arginine (10^{-4} M, ▲, n=6) or with D-arginine (10^{-4} M, ■, n=6). Vertical bars, S.E.. * or **, significant difference against control; $^{+}$ or $^{++}$, significant difference between presence of H-7 and simultaneous presence of H-7 and aminoguanidine. -E, without endothelium.

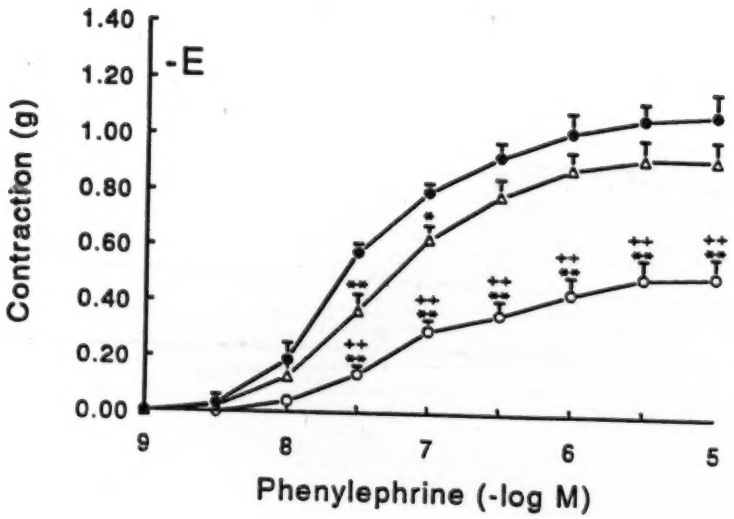

Fig.4-8. Effect of H-7 on phenylephrine-induced concentration-contraction in denuded aortic rings in the presence of methylene blue. Phenylephrine-induced concentration-response curves of denuded aortic rings in control (●, n=6), in the presence of H-7 (10^{-5} M, ○, n=6) and in the concomitant presence of H-7 and methylene blue (10^{-5} M, Δ, n=6). Vertical bars, S.E.. * or ** , significant difference against control; $^{+}$ or $^{++}$, significant difference between presence of H-7 and concomitant presence of H-7 and methylene blue. -E, without endothelium.

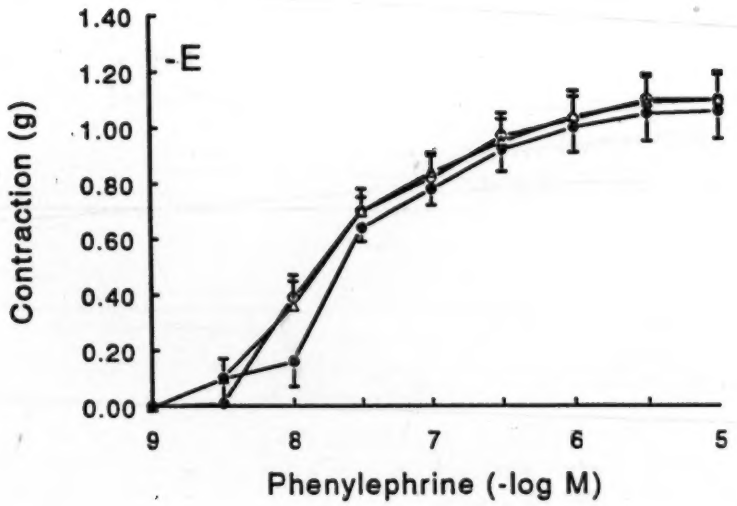

Fig.4-9. Effects of L-NNA and aminoguanidine on phenylephrine-induced concentration-contraction in denuded aortic rings. Concentration-response curves for phenylephrine in control (●, n=6) and in the presence of L-NNA (10^{-5} M, ○, n=6) or aminoguanidine (10^{-4} M, Δ, n=6). Vertical bars, S.E.. -E, without endothelium.

Table 4-1. Effect of H-7 (10^{-5} M) on phenylephrine-induced concentration-contraction in denuded aortic rings in the absence and presence of L-NNA (10^{-5} M) or in the presence of L-NNA with L- arginine (10^{-4} M) or D-arginine (10^{-4} M).

Treatment	EC_{50} ($\times 10^{-7}$ M)	Tension at 10^{-5} M PE (g)
control	0.98±0.54	1.19±0.08
H-7	2.96±0.76 *	0.59±0.06 **
H-7+L-NNA	0.76±0.13 †	1.06±0.09 ††
H-7+L-NNA+L-arg	2.05±0.92 **	0.57 ±0.04 **
H-7+L-NNA+D-arg	0.81±0.06 ▵▵	1.04±0.09 ▵▵

T_{max}= maximum tension developed, values are expressed as means±S.E. of six experiments. L-arg: L-arginine; D-arg: D-arginine; PE, phenylephrine. * or **, significant difference compared with control; † or ††, significant difference between presence of H-7 and simultaneous presence of H-7 and L-NNA; ▵▵, significant difference between presence of L-arginine and presence of D-arginine.

CHAPTER V. GENERAL DISCUSSION

The initial purpose of this study was to investigate the relationship between NO and $[Ca^{2+}]_i$ in VSM, especially the effect of NO on intracellular Ca^{2+} release. The sources of NO available in these experiments originated from vascular endothelium and sodium nitroprusside, a NO donor. Since the influence of NO on vascular contraction occurs only after NO enters vascular smooth muscle cells, it is questioned whether vascular smooth muscle has ability to produce NO. It has been well established that NOS in VSM can be induced after a long exposure to cytokines and/or endotoxin, and its activity makes a contribution to hypotension in pathophysiological processes such as septic shock (Moncada et al., 1991). However, there is little NOS activity in VSM under basal conditions (without stimulation with endotoxin and/or cytokines). It was hypothesized that NOS activity in VSM under basal conditions was inhibited probably by protein kinases (For details, see Chapter IV. 1). So the second purpose of the study was to show NOS activity in VSM after removing its inhibition by protein kinases.

These two major specific aims of our study have been achieved. The various aspects related to our experiments will be discussed in the following sections before we come to the summary and the significance of this study.

1. Roles of NO in the Regulation of $[Ca^{2+}]_i$

The vasorelaxant effect of NO has been well known (Furchgott, 1989). However, the mechanisms involved in this effect are not well understood. It is now clear that Ca^{2+}

plays a key role in the regulation of vascular contraction. Therefore, whether NO may affect $[Ca^{2+}]_i$ in VSM has been investigated in this study. Since $[Ca^{2+}]_i$ can be regulated mainly through changes in Ca^{2+} entry and intracellular Ca^{2+} release, the current study has been designed to show the effects of NO on Ca^{2+} entry and intracellular Ca^{2+} release with emphasis on the latter. KCl is well known to contract rat tail artery smooth muscle via Ca^{2+} entry through voltage-gated Ca^{2+} channels on the plasma membrane (Pang et al., 1984). In intact rat thoracic aorta, it was found that KCl elicited a sustained contraction, which was greatly attenuated by pretreatment with ACh. In primarily cultured VSMC, SNP, a NO donor, also significantly reduced the KCl-induced elevation of $[Ca^{2+}]_i$. These data suggest that NO inhibits Ca^{2+} entry through the plasma membrane, which confirms the previous results (Blatter and Wier, 1994). Our study has been focused on the effects of NO on intracellular Ca^{2+} release in VSM because controversial results have been reported by others. Using agonist-evoked phasic contractions in aortic rings in Ca^{2+}-free solution as an indicator of intracellular Ca^{2+} release, it was observed that NO selectively inhibited phenylephrine- but not caffeine-induced phasic contractions. Since phenylephrine has been known to exert its role via increasing cytosolic IP_3 concentration (Rembold, 1992) while caffeine directly stimulates ryanodine receptors (Missiaen et al., 1992). It is suggested that NO has an inhibitory effect on intracellular Ca^{2+} release through the IP_3 receptor pathway but not through the ryanodine receptor pathway. If this is case, it is significant because most endogenous vasoconstrictors, such as vasopressin (Wier and Blatter, 1991), angiotension, endothelin, histamine (Rembold, 1992), noradrenaline are able to

contract vascular smooth muscle partially via a rise in IP_3 concentration. Thus, NO may antagonize their effects *in vivo* and play a role in maintaining the homeostasis of vascular contractility. In the present study, the vascular preparation was made of rat thoracic aorta because: 1) thoracic aorta has well-developed endothelium which is maintained intact even after surgical processing; 2) VSM in thoracic aorta is a representative of large-elastic blood vessels. However, blood pressure *in vivo* is determined by cardiac output and total peripheral resistance which mainly alters with changes in contractility of arterioles. Therefore, it is desirable to employ arterioles in the future study. A difficulty encountered in the present study was to determine changes in $[Ca^{2+}]_i$ in cultured VSMCs induced by caffeine due to the loss of ryanodine receptors when cells were getting confluent (Masuo et al., 1991). Measurement of $[Ca^{2+}]_i$ in single freshly isolated VSMC from rat aorta may be responsive to caffeine.

In this study, the effects of NO on other aspects related to $[Ca^{2+}]_i$ were not investigated including extrusion of Ca^{2+} and uptake of Ca^{2+} by internal stores. Further investigation needs to elucidate their relationship.

2. NO:cyclic GMP-mediated Signal Transduction in VSMC

NO is a free radical with a half life of only a few seconds. In most circumstances, NO performs its action via binding some functional protein molecules. Among these protein molecules, soluble guanylate cyclase is the most notable. Purification of soluble guanylate cyclase has demonstrated that it contains a prosthetic heme group. NO

exhibits an exceptionally high affinity to heme iron. It has been speculated that stimulation of soluble guanylate cyclase by NO may involve the formation of a nitrosyl-heme complex, and consequently change the protein conformation. Activated guanylate cyclase thus catalyzes Mg^{2+}-GTP to form cyclic GMP (Mayer, 1994).

In this study, methylene blue, a guanylate cyclase inhibitor, was applied to test the involvement of cyclic GMP. It was found that the inhibition of intracellular Ca^{2+} release by NO was reversed by methylene blue, suggesting NO:cyclic GMP plays a role in this inhibition. In addition, it was observed that the inhibitory effect of H-7 on agonist-induced concentration-contraction was also reversed by methylene blue, indicating the involvement of cyclic GMP. In both cases, cyclic GMP was accumulated in VSM since similar results were demonstrated in endothelium-removed aorta. Therefore, these data indicate the down-regulation of vascular contraction by NO:cyclic GMP pathway.

Although the precise mechanisms by which NO:cyclic GMP interferes with the contractility of vascular smooth muscle cells are not clear, previous results have shown the possible mechanisms include dephosphorylation of the myosin light chain (Murad, 1986), interaction with the contractile apparatus (Karaki et al., 1988) and a decrease in $[Ca^{2+}]_i$ (McDaniel et al., 1992). Our data suggest that NO:cyclic GMP selectively inhibits agonist-induced intracellular Ca^{2+} release, providing a new insight into the regualation of $[Ca^{2+}]_i$ by NO:cyclic GMP in VSMC.

Most evidence from literature and our own results have demonstrated that NO exerts its regulatory roles by stimulating soluble guanylate cyclase to form cyclic GMP. However, NO itself has been found to directly activate calcium-dependent potassium

channels in vascular smooth muscle (Bolotina et al., 1994), which could cause hyperpolarization and relaxation of vascular smooth muscle independently of cyclic GMP. This may be an interesting point which we should test in the future study to find some effects of NO independent of cyclic GMP.

Although methylene blue is able to reverse the impaired contractility caused by NO, prolonged exposure of aortic rings (>20 min) to methylene blue were showed hyporesponsive to vasoconstrictors (data not shown). The reason remains unclear, but a direct damage to VSM by methylene blue following a longer exposure is speculated. If this is the case, its clinical application in the situation such as septic shock will be restricted.

3. NOS in Vasculature

Identification of EDRF as NO led to the discovery of NOS in vascular endothelium (Moncada et al., 1991). As mentioned in Chapter I, endothelial NOS is classified as isoform III, which is constitutively expressed in endothelial cells. One of the most important characteristics of this enzyme is its dependence on Ca^{2+}. Endothelial NOS can synthesize NO under basal conditions and upon the stimulation with ACh, histamine, or bradykinin. NO produced by endothelium may diffuse to the underlying VSM where it stimulates soluble guanylate cyclase to form cyclic GMP. In this study, endothelium-derived NO was shown to inhibit Ca^{2+} entry and selectively inhibit intracellular Ca^{2+} release through a distinct pathway in VSM. Thus, the endothelium is

not only a simple lining of blood vessels but also a regulator of vascular contraction. The endothelium is also a site for producing other vasoactive agents such as prostacyclin and endothelin. It is speculated that the balance of prodution of these vasoconstrictive and vasodilatory agents may be important for endothelium to maintain a homeostasis in smooth muscle contractility.

NOS also exists in VSM. In this study, pretreatment of denuded aortic rings with LPS for as long as 6 hours demonstrated an obvious hyporesponsiveness to phenylephrine, which was blocked by the NOS inhibitor, suggesting NOS proteins are expressed during the incubation with LPS. This isoform NOS is well known as inducible NOS. The phenylephrine-induced concentration-contraction in denuded aortic rings was not significantly affected by NOS inhibitors (Fig.4-9.), suggesting there is no obvious NOS activity in VSM under basal conditions. In the presence of H-7, the phenylephrine-induced contractile responses were greatly reduced. The action of H-7 could be prevented by the NOS inhibitors, suggesting it is mediated by increased NOS activity in VSM. The hyporesponsiveness in the presence of H-7 occurred rapidly and disappeared after washout of H-7, indicating NOS activity mostly comes from the existing enzyme in VSM. Since H-7 is a non-selective inhibitor of protein kinases, these results suggest that NOS activity is paobably inhibited by protein kinases under basal conditions in VSM. However, the current data were unable to show which kind of protein kinase inhibited NOS activity due to the non-selectivity of H-7. More specific inhibitors of protein kinase C is needed to elucidate this issue in the future study.

4. Summary

The conclusions of this study are summarized as follows.

5.1. NO inhibits Ca^{2+} entry through the plasma membrane in VSM.

5.2. NO selectively inhibits intracellular Ca^{2+} release induced by phenylephrine in VSM. This inhibition is mediated via cyclic GMP.

5.3. NO has no significant effect on caffeine-induced intracellular Ca^{2+} release in VSM.

5.4. The inhibitory effect of H-7 on the phenylephrine-induced concentration-contractions is mediated via NO:cyclic GMP pathway.

5.5. NO involved is mainly produced by existing NOS in VSM.

5.6. NOS activity in VSM is inhibited probably by protein kinases under basal conditions.

5. Significance

This is the first time to show that NO selectively inhibits intracellular Ca^{2+} release through a distinct pathway, providing an alternative explanation for the inhibition of agonist-induced contraction by NO. This study also for the first time describes a new mechanism by which H-7 exerts its inhibitory effect on agonist-induced tension. Furthermore, it is first found that NOS activity in VSM is masked probably due to its inhibition by protein kinases in VSM under basal conditions.

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